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CONTENTS

BROILER NUTRITION

GUT HEALTH

CALCIUM NUTRITION, BONE METABOLISM, AND EGGSHELL QUALITY IN LONGER-PERSISTING LAYER FLOCKS

D.R. KORVER[1]

Summary

I. INTRODUCTION

Commercial laying hens have been successfully selected for increasing production cycle lengths. Rather than being depopulated at 60 to 70 weeks of age, or being moulted to allow for additional production cycles, the egg industry has moved towards cycle lengths of 80 weeks or even longer. Selection for increased persistency of production and livability, skeletal health, and a slow rate of increase in egg size past peak production has resulted in commercial operations being able to achieve 500 eggs per hen at 100 weeks of age. Because of the increased lifetime output of calcium (Ca) by the hen, this extended period of production requires excellent management, including feeding the bird to optimize skeletal Ca reserves at the start of lay, and to minimize the loss of bone mass over time. The factors that contribute to skeletal health will also contribute to shell quality throughout the production cycle.

II. BONE BIOLOGY OF EGG-TYPE BIRDS

The skeleton of the pullet is comprised of two distinct structural bone tissues: cortical and trabecular. The cortical bone is the outer shell of bone tissue, whereas the trabecular bone tissue is the struts of bone that provide additional support, while minimizing bone weight as an adaptation for flight in birds. Skeletal growth of layer pullets proceeds in a similar manner as in other types of poultry and mammals with bones growing in both length and diameter. Prior to the estrogen surge associated with the onset of sexual maturity, a normal pullet with have a thick cortical shell, and well-developed trabecular struts (Figure 1A). In sexually mature hens that are laying eggs, a third type of bone (medullary bone) is formed in response to increasing estrogen levels. Medullary bone provides an additional, labile source of Ca to support eggshell formation that can be mobilized and re-deposited on a daily basis as Ca supply and demand ebb and flow with the formation of each egg.

With the onset of egg production, formation of structural bone ceases, and only medullary bone will be formed (Fleming et al., 1998b). In the weeks prior to the first egg, the bones increase in diameter (Whitehead, 2004) by approximately 20% as a means of increasing the volume into which medullary bone can be deposited. The rapid expansion of bone diameter involves both deposition of dietary Ca and P, as well as a redistribution of existing bone Ca and P mobilised from the endosteal surface (Fleming et al., 1998b; Whitehead, 2004), leaving pores in the cortical shell. As circulating estrogen levels increase in advance of sexual maturity, medullary bone is deposited as small spicules (Wilson and Thorp, 1998) lining the surface of the structural bone and also within the pores of the cortical shell (Whitehead, 2004) (Figure 1B). The deposition of medullary bone begins approximately 14 days before the first egg is laid (Hurwitz, 1964).

Medullary, but also potentially structural bone tissue is mobilized to augment the Ca coming directly from the diet in support of eggshell formation, particularly at night, when the amount of Ca coming directly from the digestive tract is limited. When the hen is not actively forming an eggshell, and dietary Ca and P supply exceeds the immediate demand, medullary, but not structural bone is replaced. Over time, the continual mobilisation and deposition of

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medullary bone leads to a more diffuse arrangement of medullary bone spicules throughout the marrow cavity (Figure 1C). Eventually, medullary bone can even fill the entire cavity of pneumatised bones (Fleming et al., 1998a). The reduction in the close association of medullary bone with the structural bone surfaces exposes the structural tissues to the action of osteoclasts. Since structural bone is not replaced as long as the hen is in lay, the cumulative effect of this erosion is a substantially decreased in structural bone mass (Figure 1C), and a greatly increased susceptibility to bone breaks (Whitehead and Fleming, 2000).

Thus, the problem of osteoporosis/caged layer fatigue in laying hens is not due to a loss of medullary bone, which then requires the hen to mobilize structural bone to support eggshell formation. Rather, it is a gradual erosion of structural bone, in spite of the presence of a large amount of medullary bone.

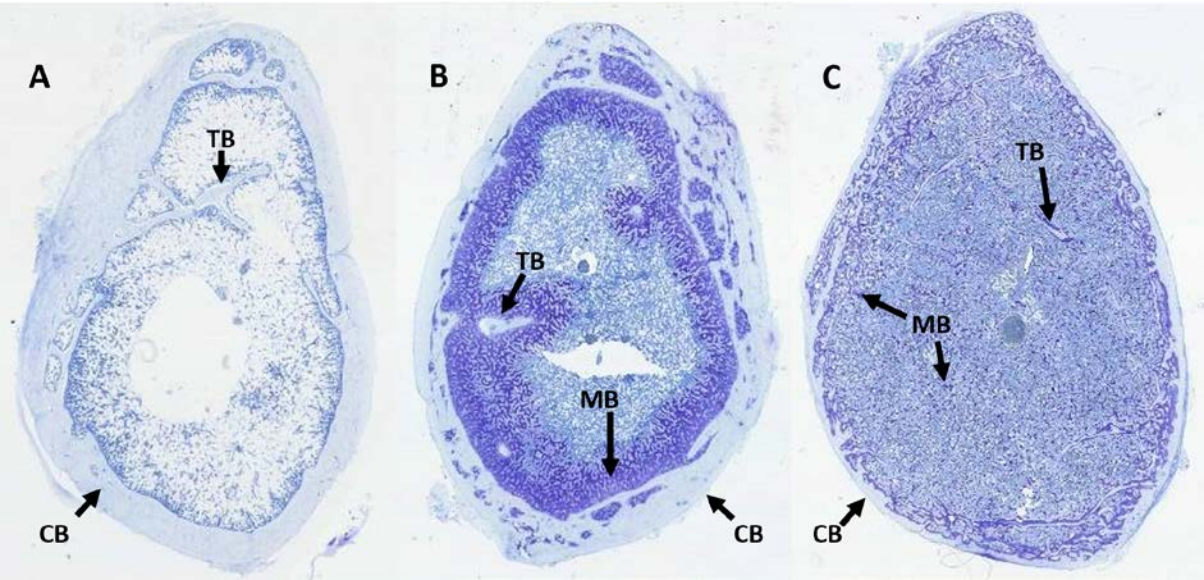

Figure 1 - Cross-sections of tibiae from laying hens of various ages. CB = cortical bone; TB = trabecular bone; MB = medullary bone. A) Layer pullet (16 weeks of age) containing only the cortical shell and trabecular struts. Diffuse staining within the cortical shell is an artifact. Structural bone (CB + TB) tissue shows very little in the way of pore formation at this level of magnification. Sexual immaturity was confirmed at time of sampling by the absence of ovary development. B) Laying hen after the first egg was laid, showing the cortical shell, trabecular struts, and medullary bone. The medullary bone is present as small spicules of bone tissue, initially deposited on the surfaces of the structural bone tissues. Pores containing medullary bone within the cortical shell are clearly visible. C) End of lay (67-week old) hen showing depletion of cortical and trabecular bone tissues, and diffuse nature of medullary bone throughout the medullary cavity. MB arrows point to some larger spicules of medullary bone.

If the hen goes through a moult, oestrogen levels decrease (Braw-Tal et al., 2004), the medullary bone is resorbed, and structural bone reserves can be replenished (Whitehead and Fleming, 2000). The gradual loss of structural bone can increase the risk of osteoporosis, and the gradual increase in egg size (Zhang et al., 2019) and the ability to deposit only a constant amount of eggshell material (Roland Sr et al., 1975) after peak production can lead to decreasing shell thickness. These two factors represent important limitations to extended egg cycles, since production rates can remain high even at older ages (Bello and Korver, 2019).

Although genetic selection for skeletal health has allowed for greatly increased productivity, as well as increased skeletal health, the high demand for Ca to support eggshell formation means that Ca nutrition and skeletal health management are critical factors from the start of the pullet phase to the end of the production cycle.

III. PULLET MANAGEMENT FOR LONG-TERM BONE HEALTH

Because structural bone deposition ceases when the pullet approaches sexual maturity (Fleming et al., 1998b), great care and attention must be paid to pullet skeletal development. Once the hen commences to lay eggs, structural bone can only be depleted as long as she remains in production. Although medullary bone can be deposited and mobilized on a daily basis, and can even accumulate over the laying cycle, it makes only a small contribution to the strength of the skeleton (Fleming et al., 1998a).

The fundamentals of proper pullet management in general are consistent with the specific requirements for optimising skeletal development at the onset of lay. Maintaining appropriate body weight and condition for age, maintaining high flock uniformity, photostimulation at an appropriate physiological state (rather than a specific age) will not only support long-term hen productivity, but also skeletal quality. The importance of excellent pullet management becomes even greater when the long-term demands placed on the bird with longer laying cycles are considered. To support the longer laying cycles currently in use in the egg industry, the pullet skeleton should be managed such that the amount of structural bone is optimized before the point at which structural bone deposition ceases, and medullary bone formation begins. At that point, the objective is to maximize the amount of medullary bone within the skeletal system.

Birds that enter production at too light a body weight are not only susceptible to a post-peak dip in egg production because of limited energy reserves; such birds are also likely to have a small skeleton and limited medullary bone reserves. Frequent weighing of birds can ensure that reductions in growth rate relative to targets are identified and rectified quickly. More frequent feeding, use of higher nutrient density diets, or midnight feeding can be used to increase nutrient intake and therefore body weight gain. Midnight feeding of pullets close to the onset of lay may interfere with the regulation of sexual maturity, and induce pullets to come into production too soon (Leeson et al., 2003). This underscores the importance of maintaining an appropriate growth rate of pullets from the beginning, rather than responding with drastic measures to a large problem. To ensure the long-term health of the birds, it is important to delay photostimulation of an under-weight flock until an appropriate body size has been reached. Pre-lay diets can be an effective means of preparing the skeletal system, but should be used with caution. Since medullary bone is not deposited until 10 to 14 days before the first egg is laid, the higher Ca level of a pre-lay diet is of no advantage to the bird before this point. Although early work with layer pullets suggested that increasing dietary Ca level well before the 10 to 14 day period in advance of the first egg had no detrimental effect on the productivity and skeletal health of laying hens (Keshavarz, 1987), such work has not been repeated with modern genetics intended for very long production cycles. Maintaining a high degree of flock uniformity is important, particularly around the onset of lay. The increase in Ca demand to support egg production is the greatest of any nutrient as the bird progresses from immature to mature. This change happens suddenly. With a very uniform flock and appropriate timing of the change from a pullet developer or pre-lay diet to the start lay diet, a high proportion of the flock will have their needs for dietary Ca met. With a non-uniform flock, the first birds that enter lay may not be receiving sufficient Ca, whereas those entering later may be receiving too much Ca, which may alter Ca metabolism (Guo et al., 2008) and impair the ability to maintain shell quality and a healthy skeleton.

IV. LAYER MANAGEMENT FOR LONG-TERM BONE HEALTH AND EGGSHELL QUALITY

At the start of the egg production cycle, the hen has the maximum amount of structural bone that she will have, until she goes through a molt. In high-producing hens, the loss of structural bone over time is inevitable, but the key is to minimize the rate at which structural bone is lost. This involves maximizing the extent to which the medullary bone acts as a protective layer on structural bone surfaces, and reducing the need to mobilize bone Ca to support eggshell formation. Therefore, optimising the supply of dietary Ca is essential for long-term skeletal health and eggshell quality. With moderate reductions in dietary Ca from breeder-recommended levels, modern laying hens can maintain high levels of egg production and shell quality, with only minimal effects on skeletal Ca reserves. This indicates both a resistance to slight reductions in Ca and available P supply and an ability to utilize bone mineral reserves to support eggshell formation (Bello and Korver, 2019). Although modern laying hens are more resistant to caged layer fatigue than in the past, their high level of production over long laying cycles can predispose them to problems with skeletal and shell quality in the long term.

An egg contains approximately 10% eggshell by weight (Pelicia et al., 2009; Bello and Korver, 2019), meaning a 60 gram egg contains approximately 6 g of shell, comprised of approximately 95% $CaCO_3$ (Nys et al., 2004), for a total of 2.3 g of Ca. This amount of Ca is obtained, in variable proportions, directly from the diet (absorbed from the gut and transported via the blood to the shell gland), or from the bone (bone mineral, both Ca and P, is resorbed by osteoclasts and the Ca transported to the blood to the shell gland. Approximately 60 to 75% of shell Ca is derived directly from the diet on shell-forming days (Driggers and Comar, 1949), and the greater the proportion coming directly from the diet, the greater the eggshell quality tends to be. Therefore, factors that increase shell quality will also reduce the need to mobilize bone Ca, and therefore will also tend to maintain skeletal health. On a daily basis, eggshell quality is negatively related to bone strength (Orban and Roland Sr, 1990). This relationship likely also impacts the long-term health of the skeleton, particularly since modern laying hens can maintain shell quality at the expense of bone mineralization (Bello and Korver, 2019). After peak production, the ability to deposit Ca onto the shell remains relatively constant (Roland Sr et al., 1975), meaning that increases in egg size after peak production will tend to result in reduced shell quality.

Dietary requirements for Ca tend to increase, and for phosphorus (P) tend to decrease as hens age. As hens age, the efficiency of Ca metabolism decreases (Wistedt et al., 2019), and increases in dietary Ca, and a widening of the Ca:available P ratio are intended to counteract this. Care must be taken not to over-feed Ca, which can reduce bone and eggshell quality (Akbari Moghaddam Kakhki et al., 2019), and interfere with exogenous phytase activity (Bello and Korver, 2019). Excess dietary P can also reduce shell quality (Miles et al., 1983). Because of its importance in Ca and P absorption from the gut, adequate dietary vitamin D activity must also be provided (Wen et al., 2019). Feeding of the vitamin D metabolite 25-OH vitamin D_3 can help to maintain skeletal and shell quality in high-producing laying hens (Silva, 2017; Akbari Moghaddam Kakhki et al., 2019).

A hen will ovulate approximately 15 to 75 minutes following oviposition (Beuving and Vonder, 1981), and the ovum will take approximately 4.25 hours to reach the shell gland (Roberts, 2004), at which point calcification takes approximately 17 hours (Hincke et al., 2012). Since hens tend to lay eggs in the morning and the early part of the afternoon (Samiullah et al., 2016; Hunniford et al., 2017), the hen can use dietary Ca and P to replenish medullary bone stores for the first 5 hours after oviposition. When the ovum reaches the shell gland, Ca demand increases dramatically to support eggshell formation. The greatest rate of eggshell mineral accretion occurs from 5 to 15 hours after the egg enters the shell gland (Hincke et al.,

2012), corresponding to the late afternoon and through the night before an egg is laid. If a hen is fed a diet containing only a small-particle Ca source such as finely ground limestone, the intestine will become devoid of a source of Ca during the night, when demand for Ca is highest. At that point, the hen will be entirely reliant on bone Ca to support eggshell formation. However, a large particle (2 to 4 mm), slowly dissolved source of dietary Ca will be retained in the gizzard for a long period of time, and Ca will be gradually released for absorption by the digestive tract, thus providing a direct dietary source of Ca throughout the night, and reducing the need for mobilization of bone Ca. A combination of 1/3 small particle, and 2/3 large particle Ca sources will provide readily available Ca to the hen when feeding begins at the start of the daytime (small particle), as well as a slowly-released source to support eggshell formation during the night (large particle).

Under conditions of heat stress, increased respiration rate can cause an increase in CO_2 loss from the bird (Franco-Jimenez et al., 2007). The reduces the pool of bicarbonate ions, and causes respiratory alkalosis, an increase in blood pH (Franco-Jimenez et al., 2007). A reduction in bicarbonate ions in the shell gland reduces the formation of $CaCO_3$, and decreases shell quality (Balnave et al., 1989).

Heat stress can also reduce feed intake, thereby reducing Ca intake and shell quality as a consequence. Midnight feeding is the addition of one to two hours of light in the middle of the dark period to all the birds to consume feed (van Staaveren et al., 2018). Birds will tend to decrease their feed intake during the day to reduce diet-induced thermogenesis, and will consume a proportion of their daily feed during the night, when temperatures are typically cooler. Thus, overall daily feed (and Ca) intake is increased. Midnight feeding can also have the benefit of providing a dietary source of Ca to support eggshell formation during the night, and reduce reliance on bone reserves (Harms et al., 1996). If the supplemental light is provided with a sufficient number of hours of dark before and after, the birds do not perceive the light in the middle of the night as the start of a new day, and regulation of egg production is not impaired.

Because the hen's Ca and available P requirements, as well as the efficiency of absorption of these nutrients fluctuate throughout the day, some researchers have begun to investigate the concept of split feeding, in which the composition of diets offered to the hen is altered to reflect the changing nutrient requirements over the course of a day (Molnar et al., 2018). More work is needed to determine whether such an approach can be successful.

In acute situations of reduced shell quality because of feed mixing errors (deficiency in Ca), reduced feed intake due to high environmental temperatures, or other factors, it may be possible to provide additional Ca in the water, or by top-dressing feed with large particle Ca. Water supplementation of Ca can be effective (Damron and Flunker, 1995), but caution should be taken, as extended use can lead to lime deposits in water lines and valves, and hens may not be able to regulate their Ca intake. Top-dressing a large-particle Ca source is labour-intensive, but allows the hens ready access to Ca. As hens have an appetite for Ca, and can regulate intake based on need (Classen and Scott, 1982), it is unlikely that hens provided top-dressed feed with will over-consume Ca.

A number of other approaches have been used to manage skeletal and eggshell quality in laying hens. Copper is a component of lysyl oxidase (Chowdhury, 1990), manganese is part of glycosyl transferases ((Leach and Gross, 1983)), and zinc is found in carbonic anhydrase (Zhang et al., 2017). These trace minerals are involved in the calcification of the collagen matrix of the shell membrane, as well as the formation of the organic matrix of bone. Organic trace minerals can have greater bioavailability than inorganic sources, and increase shell quality (Stefanello et al., 2014). Dietary fat quality can also influence shell quality. Saturated fats in the diet can form insoluble soaps that are not well digested by poultry (Atteh and Leeson, 1983), although the effect may be greater in broilers than in layers (Atteh and Leeson, 1985).

High levels of salt intake reduce eggshell formation (Balnave et al., 1989). If the water provided to hens has a high level of sodium, the addition of salt to the diet should be reduced accordingly. There is evidence that at the same level of excess, sodium from water has a greater negative impact on shell quality compared to dietary sodium.

In addition to the move towards longer egg production cycles, the global move away from cage housing to more extensive housing systems (Leenstra et al., 2016) may also present additional challenges. Although the opportunity for increased exercise may increase bone strength overall (Silversides et al., 2012; Rodriguez-Navarro et al., 2018), the greater freedom of movement may increase the incidence of keel fractures due to collisions with structures such as perches (Wilkins et al., 2011). As well, the greater exposure of the hens to environmental dust and bacteria can increase systemic inflammation (Riddell et al., 1998; Nimmermark et al., 2009; Le Bouquin et al., 2013; Roque et al., 2015), which in turn could reduce bone (Tarlton et al., 2013) and eggshell quality (Nie et al., 2018).

Because structural bone formation does not take place after the onset of lay unless the bird goes through a moult, it is imperative that skeletal quality be maintained at as high a level as possible throughout the laying cycle. Conditions which result in excessive mobilisation of bone mineral to support eggshell formation can result in increased risk of broken bones over time. This not only results in lost productivity and reduced bird welfare, but also in irreparable damage to the skeleton, as long as the hen is in lay. The negative impacts can be managed, but cannot be reversed while the hens are producing eggs. Proper pullet development, the timing of photostimulation to ensure the birds have sufficient skeletal development, and using nutritional and management factors to reduce the use of bone Ca to support eggshell formation are key aspects of maintaining skeletal health and shell quality throughout long laying cycles.

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THE BENEFITS OF MEASURING CALCIUM DIGESTIBILITY FROM RAW MATERIALS IN BROILERS, MEAT BREEDERS, AND LAYERS

P.W. PLUMSTEAD[1], M.SINCLAIR-BLACK[2], and C.R. ANGEL[3]

Summary

To-date, poultry diets have been formulated to meet estimated requirements for total calcium, while frequently also maintaining a specified ratio of total calcium:available phosphorus (AvP). In vegetable-based diets, limestone can contribute in excess of 50% and 90% of dietary calcium in broiler diets and laying hen diets, respectively. In laying hens, it is known that limestone particle size can alter the availability of calcium for shell formation and bone mineralization, and as a consequence, breed recommendations have included specifications for a minimum percentage coarse limestone grit >2mm in feed formulation. More recent research in broilers has also shown that limestone particle size, as well as the source of limestone, could profoundly alter calcium digestibility, while simultaneously having significant effects on phosphorus digestibility, and phytase efficacy. In the light of the recent studies that have documented effects of limestone quality on the digestibility of calcium and phosphorus, it was of interest to quantify differences in the quality of limestone used in commercial feedmills. With this objective, our laboratory collected 255 limestone samples from feed mills in 16 different countries on the European continent. Limestone samples were analysed for mineral content, geometric mean diameter (GMD), as well as dynamic solubility at 5,15, and 30 min for samples with <1000 µm GMD and at 30,90, and 150 minutes for grit limestone with GMD>1000 µm. While analysed calcium in limestone samples was in most cases high, there was large variation in the GMD particle size of either fine or grit limestone used in commercial feedmills. The dynamic solubility results showed that, while there was an inverse correlation with GMD and solubility at all time-points, limestone rock (geology) contributed significantly to differences in the solibilisation rates between different sources of limestone. With consideration of the recent studies that have elucidated clear effects of limestone particle size and dynamic solubility on calcium and phosphorus utilization, these large differences in the dynamic solubility rate arising from differences in the GMD particle size and geology of limestone samples used in commercial poultry feed mills can be expected to significantly alter the digestibility of calcium and phosphorus in practical diets fed to broilers and laying hens. A further consequence of this is that the ratio of digestible calcium:AvP supplied to the bird would vary between diets and/or feed mills, dependent on the quality of limestone used. This highlights that the current practice of formulating to total calcium, or maintaining a fixed ratio of calcium:AvP in feed formulation is inaccurate and that there is a need to transition to a digestible calcium system in poultry feed formulation.

I. INTRODUCTION

Calcium and phosphorus are two minerals of great concern to poultry nutritionists as a result of the relatively large quantities needed in the diet, and the adverse effects on bone formation, shell quality, and overall performance when inadequate amounts of these minerals are supplied. It is further difficult to discuss calcium supply in poultry diets without referring to phosphorus, since the dietary requirement of these two minerals has previously been shown to be interdependent (Hurwitz 1989; Coon et al., 2002; Proszkowiec-Weglarz and Angel, 2013).

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Should plasma calcium or phosphorus concentrations decrease, synthesis of 1,25-dihydroxy cholecalciferol (1, 25(OH)2D3) increases and, in turn, promotes increased intestinal Ca absorption while renal excretion is decreased (Proszkowiec-Weglarz et al., 2013; Li et al., 2017). In broilers, when dietary calcium is increased, typically by increasing the inclusion of limestone, there is also a progressive decrease in phosphorus digestibility (Li et al., 2017). The primary mechanism whereby this occurs has been thought to be directly, or via formation of calcium-phytate complexes that reduce the digestibility of phytate-bound phosphorus (Tamim and Angel, 2004; Plumstead, 2008, Jing et al., 2018). Since the utilization of calcium and phosphorus is influenced by the concentration and digestibility of the other nutrients in the diet, common practice in broilers has been to maintain a ratio of calcium to available phosphorus (AvP) when specifying requirements of calcium and phosphorus. Conveniently, this ratio has been set at 2:1 Ca:AvP in broilers (NRC 1994; Aviagen, 2014; Cobb 2018). The obvious limitation of this approach is that the interdependence of calcium and phosphorus homeostasis in the bird is driven by the amount and ratio of these nutrients provided at tissue level and hence by the ratio of digestible calcium:digestible phosphorus in the diet; and not by the ratio of total calcium:AvP supplied in the diet. In recent years, several research groups have shown that calcium digestibility in broilers can vary dramatically depending on the calcium source provided, the solubility of limestone, as well as by the source of phytate and addition of phytase (Anwar et al., 2016; Angel, 2019; Kim et al., 2019, Taylor et al., 2019).

With the knowledge that the digestibility of total dietary calcium can be significantly altered by the aforementioned dietary factors, and that the form of calcium provided and absolute amount can alter phosphorus digestibility, the specification of calcium requirements for broilers as total calcium in the diet becomes obsolete, as does the adherence to a fixed ratio of total calcium:AvP. The need to better understand how to predict variation in calcium digestibility, and the influence of calcium on phosphorus digestibility becomes even more critical when considering the high incidence of lameness and bone abnormalities observed in the industry with over 1% of commercial broilers grown to heavy processing weights affected after 5 weeks of age (Wideman, 2015). A similar case can be made for commercial laying hens for which requirements for calcium are still specified on a total basis. Particle size and solubility of limestone are known to influence the availability of calcium to the hen and can alter shell quality and bone ash (Zhang et al., 2017). These authors also suggested that the daily calcium requirement of laying hens to maintain egg shell quality should be determined based on the solubility characteristics of the limestone. While breed nutrition recommendations for laying hens do specify the supply of a portion of limestone as coarse limestone grit, potential differences in the solubility characteristics of that grit are not considered when formulating to meet the calcium demand of the hen. This observation becomes increasingly important in the context of modern laying hens where, as a result of increasing length of production cycles, optimizing the utilization of dietary calcium sources as hens age is critical to meet demands for shell formation without compromising skeletal integrity and bird welfare.

a) Variation in limestone quality and i*n-vitro* assessment thereof

In vegetable-based broiler diets, limestone can contribute over 50% of the total dietary calcium supplied to broilers, and in excess of 90% of the calcium consumed by laying hens. Given the previous observations by multiple research groups that limestone particle size and solubility can affect the utilization of calcium by broilers and laying hens, as well as phosphorus digestibility and phytase efficacy in broilers, it was of interest to characterise the observed variation in particle size and solubility of limestones used in commercial feed production in Europe to quantify differences in limestone quality used in commercial poultry diets.

II. METHODOLOGY AND RESULTS

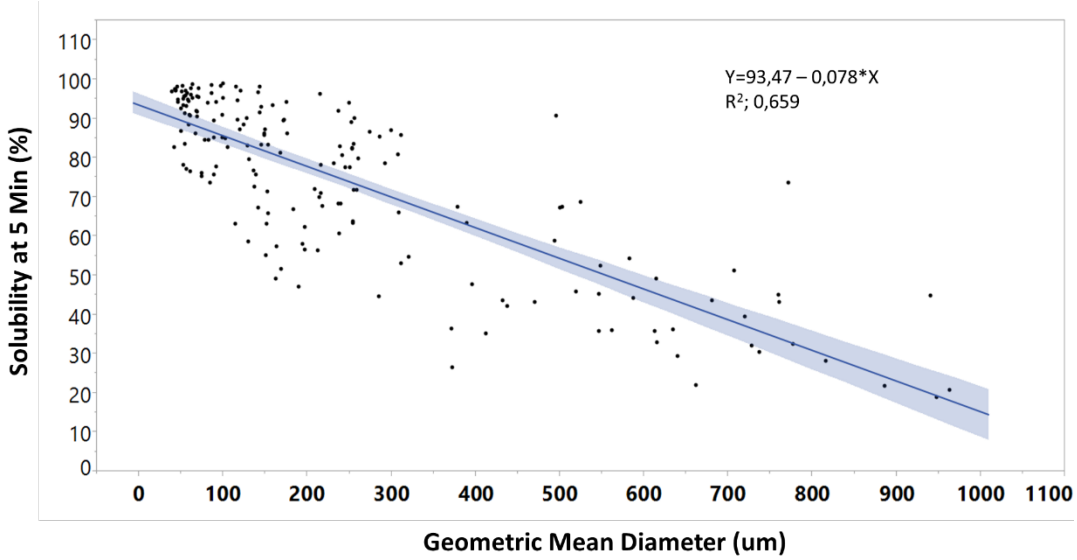
A total of 255 limestone samples were collected from feed mills in 16 different countries on the European continent. Of these, 192 samples were classed as fine limestone with an average geometric mean diameter (GMD) of <1000 µm and 63 samples as limestone grit when the average geometric mean diameter (GMD) was >1000 µm. All limestone samples were analysed for moisture, and 9 minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc). Limestone particle size was determined using a shaker and a set of 14 sieves plus the base pan using a sample of 100g of each limestone shaken for 10 minutes. The method for geometric mean diameter (GMD) of limestone particles by mass (d_{gw}) was calculated using the equations described by Wilcox et al. (1962). Solubility of limestone was determined in duplicate using the dynamic solubility assay recently published by Kim et al., (2019). For fine limestone, solubility was determined at 5, 15, and 30 minutes. Solubility of limestone grit samples was determined at 30, 90, and 150 minutes.

Table 1 - Particle size, mineral analysis, and solubility of 192 fine and 63 grit limestone samples collected from 16 countries in Europe.

	Particle size	Ca	Cu	Fe	Mg	Mn	P	K	Na	Zn	S5	S15	S30
Fine Limestone <1000 um	(um)	(%)	(ppm)	(ppm)	(ppm)	(ppm)	(%)	(%)	(%)	(ppm)	(%)	(%)	(%)
N	192	192	192	192	192	192	192	192	192	192	192	192	192
Mean	248,64	37,82	2,40	1005,27	0,28	190,42	0,02	0,02	0,02	19,97	74,23	86,87	92,89
Standard Deviation	222,70	1,93	3,39	1262,10	0,30	247,14	0,01	0,03	0,03	15,15	21,29	12,50	6,94
CV%	89,57	5,12	141,35	125,55	105,21	129,78	86,74	112,03	198,60	75,86	28,68	14,39	7,47
Minimum	38,50	30,36	0,01	42,66	0,02	4,70	0,00	0,00	0,00	0,01	18,83	39,83	62,00
Maximum	963,26	39,99	21,35	8325,53	2,86	1440,34	0,11	0,14	0,45	86,35	99,04	99,88	100,00
Limestone Grit >1000 um											S30	S90	S150
N	63	63	63	63	63	63	63	63	63	63	63	63	63
Mean	1797,43	38,26	1,97	766,28	0,26	178,96	0,02	0,02	0,02	18,39	66,89	91,29	95,20
Std Deviation	478,46	1,27	3,00	855,40	0,34	266,52	0,02	0,02	0,05	14,65	15,49	8,67	5,41
CV%	26,62	3,32	152,30	111,63	129,46	148,92	95,06	103,95	229,00	79,69	23,16	9,50	5,68
Min	1033,86	34,67	0,01	47,17	0,01	3,41	0,00	0,00	0,00	1,77	23,21	61,71	73,67
Max	3067,96	39,99	14,21	4159,40	2,65	1438,10	0,10	0,10	0,38	108,53	93,51	99,75	99,76

Particle size was calculated as geometric mean diameter by weight; S5-S150 represents the percent solubility achieved at 5, 15, 30, 90, and 150 minutes using the dynamic solubility assay of Kim *et al*., (2019).

Results in Table 1 reflect the large variation in the quality of fine and grit limestone used in commercial feed mills across Europe. Of the 192 fine limestone samples analysed, calcium levels were generally high with an average of 37.82% with only 28 samples having <36% calcium. What is particularly striking is the lack of standardization of the GMD particle size of "fine" limestone used in poultry feed. While the average GMD was 248 µm, the standard deviation of 223 µm was almost as high as the average with a CV of almost 90%. Over 48% of the fine limestone samples had a GMD below 150 µm, and 30% below 100um, reflecting he very fine nature of limestone frequently used. While there was a significant correlation between the GMD particle size and solubility of the limestone at 5 minutes (Figure 1), there are many exceptions to this generalization. For example, two limestone samples originating in the Ukraine and Poland, with similar respective particle sizes of 299 and 285 µm GMD, can have dramatically different initial solubility at 5 minutes of 87% and 45% respectively. In a similar manner, two limestone samples from different quarries in Germany had very different average GMD particle sizes of 46 µm and 250um but both reached 94% solubility at 5 minutes. Similar examples can be drawn from the grit limestone samples analyzed with samples from Germany and Turkey having similar respective GMD particle size of 2523 µm and 2587 µm yet solubilising to 73% and 42% at the first time-point of 30 minutes. These examples suggest that the geology of the limestone rock, as well as the particle size fractions, can have a profound influence on the rate of solubility of the limestone. Consequently, one cannot define an optimal particle size limestone to achieve a given rate of solubility without understanding the specific solubility characteristics of the limestone sample over time.



Geometric mean diameter by mass (Wilcox et al.,1962). Solubility determined in duplicate at 5 minutes using the assay of Kim et al., (2019).

Figure 1 - Correlation between solubility at 5 minutes and geometric mean diameter of 192 fine limestone samples collected from 16 countries in Europe.

b) Implications of differences in limestone particle size and solubility characteristics on calcium digestibility and phytase efficacy in broilers.

Several recent papers have investigated methods to determine ingredient calcium digestibility and described effects of limestone particle size on calcium and phosphorus digestibility and phytase efficacy (Proszkowiec-Weglarz et al., 2013, Anwar et al., 2016; Kim et al., 2018; Angel 2019; Kim et al., 2019). For example, in the paper by Anwar et al. (2016), fine (<0.5 mm) and coarse (1-2 mm) limestone had *in-vitro* solubility of 0.60% and 0.33% and true Ca digestibility coefficients of 0.43 and 0.71, respectively. These findings demonstrated for the first time in broilers that a positive correlation existed between limestone particle size and *in-vivo* calcium digestibility, with calcium digestibility being negatively correlated to limestone solubility. Kim et al. (2018) also showed fine limestone (0.75 µm) to have a higher *in-vitro* solubility that was supported *in-vivo* by a higher gizzard pH in 28-d old broilers fed diets with 0.8% or 1% calcium. However, in that study, the adverse effects of the fine, more rapidly soluble limestone on calcium digestibility were only observed when diets had a lower calcium level (0.6%) and no phytase. Further publications by the same group, Angel, (2019), and Kim et al. (2019) have subsequently provided greater insight into the impact that differences in limestone solubility arising from different sources (geology of limestone), or different particle size, can have on calcium digestibility in broilers. When comparing limestone from the same source, a reduction in particle size from 0.8 mm GMD to 0.15 mm GMD reduced standardized ileal digestibility (SID) of calcium from 49.2% to 38.1% (Angel, 2019). Importantly, in the paper by Kim et al. (2019), differences in GMD particle size alone could only explain <40% of the observed differences in calcium digestibility from limestone; and differences in limestone geology and physical/chemical characteristics were equally important to particle size in their potential effects on calcium digestibility. This observation is supported by our findings in the European limestone survey above, that limestone particle size alone was not able to adequately explain the large variation observed in *in-vitro* solubility between different samples of limestone. Initial models by Kim et al. (2019) further showed that differences in calcium digestibility between limestones could be explained by the extent of solubility achieved at 15 and 30-minutes *in-vitro*, highlighting that limestone must be solubilised in the

proventriculus/gizzard in order to be digested. In addition to differences in limestone characteristics, the group at the University of Maryland has also shown the phytate source to alter digestibility of calcium from limestone, with phytate from corn tending to be more reactive with calcium from limestone than when diets contained a mixture of corn and soybean meal (Angel, 2019).

While not the main focus of this paper, one of the outcomes of the recent research focus on calcium digestibility was that the solubility kinetics of limestone could potentially alter phosphorus digestibility to a far greater extent than the observed differences in calcium digestibility (Kim et al., 2018; Angel 2019; Kim et al., 2019; Taylor et al., 2019). Using one example from our laboratory, Taylor (2019) evaluated three different sources of limestone that had been standardized to a 0.8mm GMD, or simply included in the same diet at the commercial particle size supplied by each limestone company. For each limestone, increasing the particle size and thereby slowing down the rate of solubilisation increased phosphorus digestibility. Of equal importance was that phytase efficacy and in-particular, matrix values from phytase, were significantly affected by the source of limestone used in the diet and is supported by the previous findings of Kim et al., (2018).

The significance of these and other research on limestone and calcium digestibility in practical broiler diet formulations still needs to be better understood. One of the reasons why we may not have seen large differences in broiler performance when commercial feed mills use different limestone sources is that analysed calcium and phosphorus levels in commercial broiler grower and finisher diets are typically far above the birds' requirements determined in more recent research (Jiménez-Moreno, 2013). However, given environmental pressures on reducing phosphorus excretion in broiler manure in some regions, as well as the recent trend of reducing calcium levels in commercial broiler grower and finisher diets, the frequency whereby broiler performance is compromised as a result of not accurately defining the amount of digestible calcium supplied in diets of broilers will likely increase in the future.

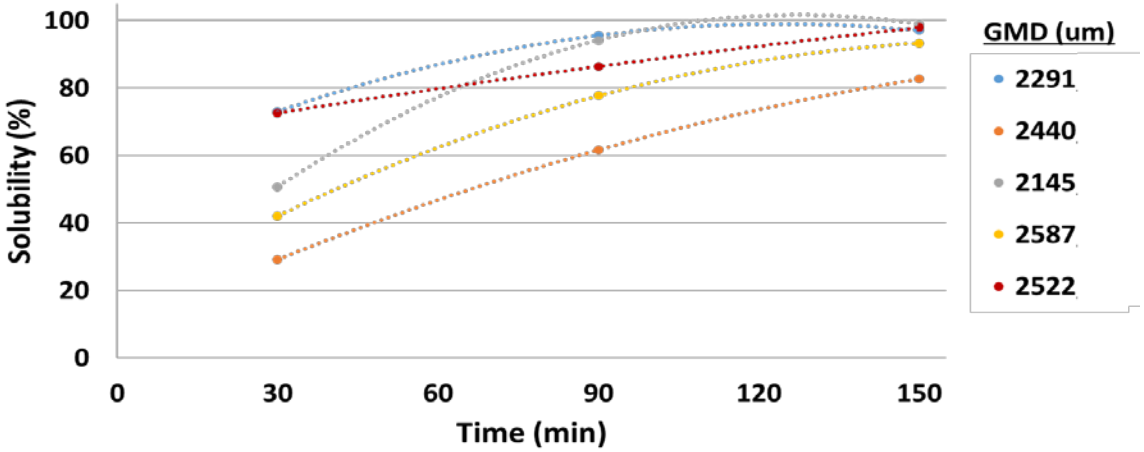
c) Implications of calcium digestibility and effects of limestone particle size and solubility characteristics in laying hens and broiler breeders.

Over short periods, commercial laying hens and broiler breeders are particularly resilient to small variations in calcium supply *vs*. that required for egg formation. This is as a result of homeostatic mechanisms designed to regulate the supply of calcium for shell formation in times of excess or deficiency relative to demand. For example, should the diet contain insufficient digestible calcium to maintain the calcium demand for shell formation, the subsequent reduction in ionized blood calcium (iCa) stimulates parathyroid hormone (PTH) that is designed to elevate blood iCa to the hen (Singh et al., 1986). However, the elevation of blood iCa through elevated PTH is in-part achieved through the initiation of medullary bone breakdown. If the dietary calcium deficiency persists over extended periods, the hen will start to break down structural cortical bone (Whitehead, 2004). Therefore, the trade-off that occurs when insufficient digestible calcium is provided is that the hen compromises the structural integrity of her skeletal system, which results in a shorter productive life of the hen since the scavenged cortical bone is not replaced. This is a much greater problem in commercial hens for table eggs than it is for broiler breeders, both as a result of the shorter laying cycle and lower egg output of breeders.

A further issue that arises when laying hens utilize calcium from their bone reserves, is that eggshell quality decreases as the hen utilises more calcium from her bones (Sauveur and Mongin, 1983). Eggshell breakages form a large proportion of economic losses incurred by egg producers (Hamilton et al., 1979). Therefore, the determination of calcium digestibility

values for ingredients may pose a solution to mitigate the effects of inaccurate calcium formulation for laying hens.

Since limestone as an ingredient contributes in excess of 90% of the calcium consumed by the hen, any variation in the digestibility thereof can significantly alter the supply of calcium at tissue level. The i*n vitro* solubility of limestone at a single time-point has been reported to be inversely related with its *in vivo* solubility (Zhang and Coon, 1997). Larger limestone particles with a lower solubility would reside in the gizzard longer, thereby increasing the *in vivo* Ca availability and improving egg shell quality and bone mineralization in the hen (Rao and Roland, 1989; Zhang and Coon, 1997; Zhang et al., 2017; Sinclair Black, 2019). However, in contrast to these observations in laying hens, Bueno et al., (2016) observed no effects of limestone particle size on egg characteristics and performance of broiler breeders post-moulting. However, this research was done in post-moult broiler breeders from 74-83 weeks and may not have been long enough feeding period to show effects of limestone particle size. Consequently, more research is therefore required to elucidate if limestone particle size is influential on calcium utilisation in broiler breeders that are restrict fed a single meal a day. In laying hens, there is little doubt that coarser particle limestone with lower solubility will be beneficial the for saleable egg output, and the maintenance of bone integrity. Based on this, commercial recommendations for laying hen nutrition have incorporated the provision of a certain percentage of total dietary limestone to be provided as "grit" with the balance of limestone being supplied as fine or powdered limestone. While the minimum/maximum particle size of grit was specified between 2mm and 4mm (Hyline, 2013; Decalb-Amberlink 2019), this does not consider potential variation in the solubility of grit as a result of observed differences in the geology or origin of the limestone. As was shown in the survey of limestone grit samples (Table 1), there was a large variation of what particle size is defined as "grit" in Europe, and equally large variation in the rate of solubility over time. This is illustrated in Figure 2 that depicts the rate of solubility of 5 samples of limestone within a narrow range in GMD particle size of 2145-2522 µm.



Geometric mean diameter by mass (Wilcox et al.,1962). Solubility determined in duplicate at 30, 90, and 150 minutes using a modified assay of Kim et al., (2019).

Figure 2 - Dynamic solubility of limestone grit sampled from 5 countries in Europe

The objective of supplying grit to laying hens is to reduce the rate of limestone solubilisation, and therefore slow down the delivery of dietary calcium to coincide with the period of shell formation later in the day / evening. The data in Figure 2 show that a limestone with 2522 µm can potentially solubilize in excess of 75% within 30 minutes *in-vitro* at a pH of 3.0. In contrast, a second limestone with almost identical GMD (2587 µm) only reached 42% solubility at 30 min. These two limestones would, therefore, potentially deliver calcium to the

hen at very different rates and be less or more beneficial in meeting the objectives of a slow release of calcium for egg shell formation. Further, one of the objectives of still providing a proportion of "fine" limestone in laying hen diets is to meet the rapid demand for calcium required to replenish the medullary bone supply in the morning. Based on many of the limestone grit samples analysed solubilizing over 50% at the first time-point of 30 minutes, one could speculate if there is indeed a need for a separate source of "fine limestone", and is the subject of ongoing research in our laboratory.

In terms of determining calcium digestibility in laying hens, a further aspect that should be taken into account is the hens ability to rapidly alter calcium digestibility based on the time of day, or rather the physiological demand for blood iCa for shell formation. This was recently investigated in our laboratory with results showing that ileal calcium digestibility changed from 54.61% at 3h post oviposition to 64.77% 11h after oviposition, and during the time of peak shell formation (Sinclair-Black, 2019). This finding has several implications, foremost that when determining calcium digestibility for the purpose of developing matrix values for feed formulation, the timing relative to oviposition time of the hen can dramatically alter results. Further, from a commercial perspective, while calcium requirements of the hen are specified as total Ca to be supplied/day, the timing of when that calcium is supplied will greatly affect its utilization and subsequently the absolute total amount required. This again reiterates the limitations of continuing to specify calcium requirements and feed formulation on the basis of total calcium.

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TRIBUTYRIN CAN IMPROVE EGG PRODUCTION RATE AND EGG QUALITY IN VERY OLD LAYERS BY INCREASING THE DIGESTIBILITY OF CALCIUM AND PHOSPHORUS

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In the past few decades in South East Asia (SEA), with a moderate increase in the layer hen population, egg production rate and lifespan have greatly improved. However, when compared with the global egg production per capita (less than 7 vs. more than 10), there is still a significant opportunity to further increase egg production in this area. In SEA, the top challenges for the layer industry are egg quality and egg production rate, especially for old flocks. In old layers, especially after 50 weeks of age, the aging of the intestinal tract can negatively impact nutrient digestion and absorption (Sakdee et al., 2018). As a well-known supplement for improving intestinal development and gut health, butyrate based products have been widely used in the layer industry since late last century. Until very recently, tributyrin (TBU) as the carrier of both butyrate and monobutyrin (MBU), was regarded as an ideal replacement for, or more accurately an upgrade of, the generic butyrate-based product (Bedford and Gong, 2018). Within the gastrointestinal tract of farm animals, one unit of tributyrin is hydrolyzed by lipase into, approximately, two units of butyrate ions and one unit of α-MBU (Perstorp, internal data). The benefits from butyrate will be retained in TBU, and more functions from α-MBU. Therefore various tributyrin-based products (e.g. ProPhorce^{TM} SR130 from Perstorp) were used to improve the performance of layers in terms of egg production and egg quality.

Twelve pens of 144 layers at 86 weeks of age (Bovan White Leghorn) were used to evaluate the effect of a butyrate-based product (SR130) on egg production rate and quality. Two groups of layers were fed *ad libitum* with either a standard diet (SD) or SD with SR130 supplement at the level of 50 grams/ton feed. After 14 weeks, i.e. when the layers were 100-week old, feed efficiency, egg production rate and egg quality were measured. During the duration of the trial, the group treated with SR130 (SRT) had a consistently significantly higher ($P < 0.05$) average daily feed intake (104.9 g/hen/day vs. 102.2 g/hen/day) than the control group (CTR). Similar trends for average egg production rate (66.68% vs 66.02%) and average egg weight (66.92g vs. 65.93g) were also observed in the two groups with significant differences ($P < 0.05$). Furthermore, as of week 4 of the trial, we found a continuous significant increase of egg shell strength in SRT as compared with CTR ($P < 0.05$). This was partially due to the significant increase ($P < 0.05$) in both the calcium digestibility (from 25.91% to 35.94%) and phosphorous digestibility (from 25.75% to 28.43%) in SRT as compared with CTR.

It is well documented that butyrate can have positive effects on gut morphology and gut integrity in farm animals (Bedford and Gong, 2018). The additional functional molecule MBU, released from TBU, can synergistically balance the microbiota with butyrate and it can also contribute to capillary network development. With the combination of butyrate and MBU derived from TBU, egg production rate and egg quality were improved in the very old laying hens.

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USE OF COMMERCIAL PREMIXES WITH LOW LEVELS OF ORGANIC TRACE MINERALS ON EGG PRODUCTION PERFORMANCE, AND EGG SHELL CHARACTERISTICS IN LAYERS

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Summary

Common industry practice is to supply trace minerals well above published recommendations. An experiment was conducted to examine the effects of two commercial premixes with common levels of trace minerals (Cu, Mn, Fe, Zn) in inorganic form or reduced levels of trace minerals in organic form on production performance of laying hens, egg weight, egg and shell quality. The study showed that the use of low levels of organic trace minerals in the diets had no impact on egg production or feed conversion ratio. However, birds fed organic trace minerals had heavier eggs in the first 10 weeks (0-10weeks) and increased eggshell strength measured in week 20 ($P<0.05$). In addition, there was a tendency for improved egg quality after storage ($P = 0.07$) in diets with organic trace minerals.

I. INTRODUCTION

It is well known that essential trace minerals such as zinc, copper, manganese or iron are involved in hormone, enzyme, carbohydrate protein or nucleic acid metabolism. Insufficient levels of trace minerals in a diet can lead to poor health and subsequently poor performance (Smith & Akinbamijo, 2000). Trace minerals are a very small part of the overall cost of a diet which allows nutritionists to use high safety margins (2-5x NRC levels) in their feed formulations. As such, trace mineral requirements for modern poultry production is poorly researched and most commercial diets include levels that are well above the NRC recommendations published over 25 years ago (Leeson, 2005). More recently, a better understanding of the correlations between trace mineral use and excretion into the environment, trace mineral loads and antimicrobial resistance and the increase in contamination with toxic heavy metals alerted the European Feed Safety Authority (EFSA) to critically review the use and overuse of trace elements (Brugger & Windisch, 2015, Elliott et al., 2017). In addition, a better understanding of the role of inorganic trace minerals in feed, in particular the negative interactions with vitamin or endogenous feed enzymes, raised serious doubts about current practices. The availability and use of more bioavailable organic trace minerals have reignited the debate on appropriate levels of trace minerals in poultry diets. Work in broilers showed that using low levels of organically bound trace minerals had no impact on broiler performance but significantly lowered excretion rate of these minerals (Nollet *et al.*, 2007). Similarly, a study by Abdallah et al. (1994) showed that removing all supplementary trace minerals from layer diets for 10 weeks had no effect on laying performance of hens, but significantly decreased egg shell weight. Despite the fact that organic trace minerals are protected from interaction between minerals, it is important to understand the limiting levels of each mineral. A series of experiments by Bao et al. (2010) established that Zn is the first limiting trace mineral among Cu, Mn, Fe and Zn. These limitations need to be taken into consideration in commercial feed formulations. The objective of this study was to determine the effects of a commercially available layer premix with reduced levels of trace minerals in organic form.

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II. MATERIALS AND METHODS

One hundred and forty-four ISA brown laying hens (64 weeks of age, standard vaccination program at rearing) were randomly allocated in groups of 4 hens (n=4) and housed in an experimental layer facility at the University of Queensland, Gatton in standard layer cages (650cm^2/bird stocking density). Birds were fed a commercial layer diet for 3 weeks. At the start of the experimental period, cages were divided into two groups of uniform egg production and allocated to experimental diets with either inorganic trace minerals at industry levels (ITM) or organic trace minerals at low levels (OTM) (Table 1) with 18 replicates of 4 hens each.

Table 1 - Premix specifications - Trace mineral levels.

Ingredient (ppm)	NRC	Breed standard*	Commercial levels of inorganic trace minerals (sulfate/oxide)	Commercial levels of organic trace minerals (Proteinate**)
Cobalt	0.25	0.2	0.2	0.25
Copper	6	10	8	4
Iron	10-80	80	60	12
Manganese	40-80	85	90	30
Zinc	50	80	60	30
Selenium**	0.3	0.3	0.3	0.3

* (Hendrix Genetics, 2019)
** Bioplex® minerals (Alltech Inc.), ** Sodium selenite or Selenium yeast (Sel-Plex®, Alltech Inc.)

During the 30-week experimental period - 67 to 97 weeks of age – per cage daily egg production, egg weight and feed intake was measured. At the end of week 10, 20 or 30 respectively one egg from each cage was selected (n=18) and eggshell breaking strength was measured using an Egg Force Reader (Egg Multi Tester-EMT-5200, Robotmation Co. Ltd Tokyo Japan). Quality of eggs stored at room temperature was measured at the end of the experiment (one egg per cage, n=18) using an Egg Analyzer TH (EMT-5200). SAS systems (SAS Institute Inc., 2001) was used to perform the statistical analyses used in this study. Data were subjected to analysis of variance (ANOVA). Least significant differences between means was used to test for the probability of significant ($P<0.05$) differences between means.
The study was conducted to the standards set by The University of Queensland Animal Ethics Committee and Australian Model Code of Practice for the Welfare of Animals –Domestic Poultry 4th Edition, 2002 (UQ study number C03360-001).

III. RESULTS AND DISCUSSION

Overall performance throughout the study was excellent and slightly above the expected target production for the breed (Hendrix Genetics, 2019) . The source and level of trace minerals had no effect on laying performance or feed conversion although laying performance of birds fed organic trace minerals remained numerically higher during the experimental period (Table 2). Hence, trace minerals from organic sources supplemented at levels notably lower than commercial levels can maintain optimal growth and production performance.

These findings are in line with work by Stefanello et al. (2014) which showed that feeding similar levels of Mn, Zn, and Cu resulted in higher eggshell thickness compared to inorganic sources at the same level. Stefanello et al. (2014) showed that mineral levels had a quadratic effect on egg weight and eggshell quality. These researchers were also able to demonstrate a significant increase in egg weight ($P<0.05$) in birds supplemented with organic trace minerals and linked the increase in egg weight to improved eggshell thickness due to

higher mineral supplementation. Similarly, work by Rutz et al. (2004) reported a significant interaction between the dietary effect of trace mineral source and eggshell thickness when Mn and Zn were supplemented at similar levels to the current study. Eggshell thickness and egg production were significantly improved when layers were supplemented with organic trace minerals ($P<0.05$).

It is well known that trace minerals are involved in various aspects of the formation of eggshell and an increased availability in Mn, Zn and Cu results in higher shell strength and better quality in the eggshell formation (Stefanello et al., 2014). Despite the reduced levels of trace minerals in the feed, supplementation of organic trace minerals had no negative impact on shell quality. In the current study, the increase in egg weight and the eggshell strength in birds fed organic trace minerals was equal to or improved compared to supplementation with inorganic trace minerals. Haugh units in eggs from birds fed organic trace minerals remained higher ($P<0.07$) when stored for 14 days at room temperature. Although it is inevitable that Haugh units will decrease over time, the positive effect of organic trace minerals has been previously reported by Batista et al. (2017).

Table 2 - Effect of two sources of trace minerals (Inorganic trace minerals – ITM; organic trace minerals -OTM) on production performance and egg quality of laying hens (67-97 weeks of age).

Variable	ITM	OTM	LSD*	P-value
Egg production (Hen Housed %)				
0-10 weeks	89.3	91.9	3.81	NS
0-30 weeks	80.5	82.2	5.40	NS
Egg weight (g/egg)				
0-10 weeks	65.3^{b}	67.0^{a}	1.69	<0.05
0-30 weeks	65.8	66.9	1.63	NS
FCR				
0-10 weeks	2.07	1.98	0.10	0.10
0-30 weeks	2.12	2.08	0.10	NS
Egg Shell break force (N/kg)				
Week 10	3.32	3.06	0.47	NS
Week 20	2.92^{b}	3.41^{a}	0.46	<0.05
Week 30	3.28	3.12	0.51	NS
Evaluated at 97weeks of age:				
Egg quality 7 days stored (~28°C)				
Albumen height	4.16	4.48	1.10	NS
Haugh unit	56.22	55.94	12.81	NS
Egg quality 14 days stored (~28°C)				
Albumen height	3.07	3.45	0.59	NS
Haugh unit	39.27	47.11	8.78	0.07

a,b,c Values with unlike superscripts differ significantly ($P<0.05$), * Least Significant Difference (LSD)

The results of the present study show that the use of commercial premixes with organic trace minerals at lower levels than common industry practice has no negative impact on laying performance but has a beneficial effect on eggshell and egg quality.

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PRINCIPLES AND PRACTICAL FEEDING OF THE MODERN LAYING HEN UNDER ALTERNATIVE PRODUCTIONS SYSTEMS: EFFECTS ON BIRD WELFARE AND PRODUCTION

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Summary

Conventional cages were banned in the European Union in 2012, with a clear trend since then to use new alternatives for egg production in all the countries. The new systems have 3 main objectives: a) meet the social demands of the society (food safety, poultry welfare and sustainability), b) maximize egg production while keeping production cost under control and c) maintain egg quality throughout the egg cycle. The ban on the use of conventional cages, moulting the birds, and beak trimming, together with the implementation of the new systems, have changed bird behaviour and thus, flock management. Under the new conditions, hens are attracted to activities other than eating and drinking, with effects on behaviour, feed intake and flock uniformity. Moreover, the restriction of use of some pharmaceutical products compromises the control of parasites and diverse bacterial diseases. As a consequence, strategies to maximize egg production are a challenge for poultry nutritionists and veterinarians. New thinking and knowledge in certain areas of nutrition such as a) feed form, particle size and texture of the diet (feed intake, behaviour), b) levels of inclusion of fibre and crude protein in the diet (behaviour, litter quality, sustainability, liver health) and c) control of mineral content of the diet (Ca, P and electrolytes), are needed.

I. INTRODUCTION

Consumer demands and the pressure of supermarket chains are changing rapidly the way eggs are produced. Enriched cages are not considered a sound alternative to improve animal welfare standards in the developed countries. Consequently, the industry is moving hens from battery cages towards alternative systems, such as deep litter and aviary barns, with or without access to an outdoor area. In addition, organic production with non-beak trimmed hens, under free range conditions, is gaining popularity. In 2018, the percentage of hens reared under these systems in key countries of the European Union (EU-28) is shown in Table 1 (European Commission, 2019).

Table 1 - Alternative systems for laying hens by country. European Union-28, 2018.

Country	Hens		Cages[1]	Barn	Free range	Organic
	× 10^6	% of total	(%)	(%)	(%)	(%)
Germany	53.5	12.8	6.5	62	19.5	12.0
Italy	50.0	12.0	54.7	38.1	3.3	4.0
Poland	48.5	11.6	84.5	11.2	3.6	0.7
UK	46.6	11.2	35.2	5.2	56.9	2.7
France	46.5	11.2	60.8	8.0	21.3	9.9
Spain	43.6	10.4	82.3	9.4	7.4	0.9
Total	417	100	50.4	28.5	15.7	5.4

[1] Enriched (750 cm^2/hen) European Commission, 2019

Of note is the growing interest for organic and free-range production systems in Northern Europe as compared with the relative high percentage of caged hens in the Eastern

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and Southern parts of Europe. Historical data on the production systems in Spain, a key country in egg production in Europe, are shown in Table 2. From 2016 to 2018 the percentage of caged hens decreased from 92.9 % to 82.3 %. A greater rate of change is expected for the next 2-3 years.

Table 2 - Alternative systems for egg production in Spain. Historical data.

	2016	2017	2018	Trend[1]
Enriched cages, %	92.9	88.0	82.3	↓↓
Barn[2], %	2.4	6.0	9.4	↑↑
Free range, %	4.0	5.0	7.4	↑
Organic, %	0.6	1.0	1.0	↑

[1] Less than 40 % hens expected in cages by 2025 European Commission, 2019
[2] Mostly, aviary type

Egg production in alternative systems requires expertise on behaviour and management of the birds. Nutrition is, after management, the main factor to control in modern egg production (Ruhnke, 2015). Areas of concern (and requiring improvement) in practice, include a) maximize feed intake (FI), especially in small frame hens exposed to the environment and with high physical activity, b) increase BW uniformity of the flock to decrease safety margins in feed formulation, c) reduce the incidence of feather pecking and cannibalism to increase liveability and animal welfare and d) produce high quality eggs of adequate size for the first months and good shell quality for the last months of the egg cycle (van Krimpen et al., 2005; Ruhnke, 2015; Lohmann, 2017; van Emous and van Krimpen, 2019; Kaukunen and Valros, 2019; Iqbal et al., 2019).

II. MAXIMIZING FEED INTAKE

The goal of all the alternative systems is to optimize hen production to reach similar performance to that of caged hens at similar cost. In general, hens reared under alternative systems lose weight during the first 2 to 3 wk of the pre-peak period (entry into the barn) and thus, maximising body weight (BW) of the pullets before the onset of egg production is a sound management and nutritional strategy. Maintenance requirements are higher for hens in alternative systems than for hens in cages, especially in aviaries with access to an outdoor area. At low ambient temperature, hens consume energy to maintain body temperature resulting in poor feed efficiency, a problem that is aggravated in flocks with poor feather cover. Peguri and Coon (1993) reported differences in feed intake (FI) from 82 g/d in well feathered hens under heat stress conditions (33.9 ºC) to 147 g/d in totally de-feathered hens at 12.8 ºC (Table 3). On the other hand, good feather cover might reduce energy intake below requirements of laying hens under hot weather conditions. Under these circumstances, an increase in the energy content of the diet, or changing feed form from mash to crumbles, might help to solve the problem.

Modern lines of laying hens have been selected for years for reduced frame size and for increased persistency in egg production. Thus, feed efficiency has improved but the intake capacity of young hens, under stressful situations, might not be sufficient to meet nutrient requirements. As a consequence, hens lose BW with a subsequent reduction in egg size and egg production. Data in Table 4 illustrate that maintenance needs of aviary hens with access to the outdoors might be up to 10 to 15 % higher than those of caged hens. However, it should be noted that the energy requirements for egg production with mash diets are similar for all systems.

Table 3 - Influence of ambient temperature and feather cover on feed intake in Single Comb White Leghorn hens.

T ºC	Feather cover, %			Average
	100	50	0	
12.8	108	128	147	128
23.9	105	112	125	115
33.9	82	91	99	90
Average	98	110	124	

Peguri and Coon, 1993

Table 4 - Energy requirements of laying hens for optimal egg production. A comparative study.

	Egg production	Physical activity	Ambient T ºC	Energy[1] needs
Cage	3 +	+	+	+
Barn indoor	3 +	2 +	+	2 +
Aviary indoor	3 +	3 +	+	3 +
Aviary free range	3 +	4 +	3 +	4 +

Three strategies used to overcome the problem of low FI at the start of the egg cycle under stress conditions are 1) apply the empty feeder technique, 2) improve feed structure of the diet and 3) increase the fibre content of the pullet diet from 10 to 17 wk of age. The empty feeder technique consists of training the pullets (after 4 to 5 wk of age) to consume as much feed as possible. Immediately after the lights go on, an extra amount of new feed is placed in the feeders as needed. At mid-day, pullets are forced to clean the feeders, consuming all fines left in the feeders for 60 min. Then, new feed is supplied 2 to 3 times during the afternoon, until the lights go off. A similar strategy should be used in the laying hens to ensure that at least 60 % of the feed (and Ca) is consumed in the afternoon, resulting in an increase in BW gain and shell quality during the entire egg cycle.

Feed form, average mean particle size (MPS) and feed uniformity affect FI in all types of birds (Röhde et al., 2014). Usually, pullets and laying hens are fed mash diets because of cost and the apparent lack of benefit of pelleting (or crumbling) on performance. The use of crumbles, however, might be recommended under some circumstances, such as pullets from 0 to 5 wk of age, and light hens at the start of laying cycle (15 to 25 wk of age). Feeding high quality crumbles during the prestarter period increases BW gain and uniformity as well as feed efficiency (Frikha et al., 2009a,b; Guzmán et al., 2015). Also, young hens fed low energy diets, under hot weather conditions, might benefit of the use of crumbles during the pre-peak period. However, feeding crumbles jeopardizes the development of the gastrointestinal tract (GIT), reducing future voluntary FI, a current situation at the initial stages of the egg cycle (Saldaña et al., 2015a, b). Under these circumstances, the inclusion in the diet of insoluble fibre sources might help to overcome the problem.

It is accepted that laying hens show a preference for consuming coarse vs. fine particles (Portella et al., 1998; ISA Brown, 2008; Safaa et al., 2009) which, in turn, might result in an increase in egg production and egg weight (Table 5). The data available, however, do not show a clear, linear, positive relation between MPS of the diet and voluntary FI and egg production of laying hens under commercial practices (Hamilton and Proudfoot, 1995; Ege et al., 2019). Herrera et al. (2017, 2018a) reported a preference of laying hens for coarse particles but the preference did not result in an increase in hen production (Tables 6 and 7).

Table 5 - Influence of particle size of the diet on feed intake and egg production in brown laying hens.

	Standard diet (%)	Fine diet (%)
Mean particle size, mm		
< 0.5	9	31
0.5 – 3.2	81	69
> 3.2	10	0
> 1.6	65	21
Egg production		
Egg rate (%)	94	91
Feed intake (g/d)	118	114
Egg weight (g)	63.3	62.7

ISA, 2008

Table 6 - Preference of brown egg laying hens for the coarse particles of the diet[1] (geometric mean diameter of the feed remaining in the feeders).

Screen size (mm)	Sampling time[2]		Difference
	06:00 am	14:00 pm	
4	1050	900	150
6	1102	881	221
8	1313	991	322
10	1386	1017	369
12	1494	1061	433
SEM (n=10)	11.1	16.0	
P-value (L)	***	***	

[1] Average of maize and barley diets
[2] Data at 08:00, 10:00, and 12:00 am, not shown

Herrera et al., 2018a

Table 7 - Effects of mean particle size of the diet[1] on production of brown egg laying hens from 17 to 49 wk of age.

Screen size[2,3] (mm)	Egg rate (%)	ADFI (g)	Egg mass (g/d)	FCR (g/g)
4	91.7	112.6	56.5	1.99
6	93.0	112.7	57.7	1.95
8	93.0	112.9	57.5	1.96
10	92.9	112.9	58.0	1.95
12	93.1	113.2	58.0	1.95

[1] Average of the maize and barley diets
[2] Hammer mill
[3] $P < 0.05$ for all variables when comparing 4 mm vs. all others

Herrera et al., 2018a

Similarly, Herrera et al. (2018b) did not show any benefit in terms of egg production when the main cereal of the diet was ground with a 6- or 10- mm screen. We hypothesised that the enhanced FI observed when hens are fed mash diets, might not depend exclusively on the proportion of coarse particles. Data from our lab suggest that poultry in general, and hens in particular, decline to eat fines and do not necessarily eat "preferentially" coarse particles. In this respect, hens eat more of diets ground with a roller mill than of diets ground with a hammer mill. The main difference on the MPS produced by these 2 grinding processes is not the size of the resulting particles, but the lower uniformity and higher percentage of fines produced by the hammer mill. In fact, two strategies used to increase FI under hot weather conditions, are the supplementation of the diet with fat and an increase in the percentage of coarse calcium carbonate particles of the diet. Adding fat to mash diets agglomerates the fine particles present in the feed, increasing MPS and improving FI. Also, it is recommended to use at least 70 % of

the calcium carbonate of the diet as coarse particles (2-4 mm Ø) to improve calcium (Ca) digestibility and feed structure. Similarly, a by-pass of the hammer mill of ingredients already ground (i.e., soybean meal), reduces the percentage of fines and improves FI in commercial laying hen operations.

III. DIETARY FIBRE

Diets for modern laying hens are formulated to maximise FI, especially at the start of the laying cycle. It has been assumed that dietary fibre (DF) reduced FI as well as the digestibility of other components of the diet, resulting in digestive disorders in broilers and young pullets. As a result, diets for poultry are often formulated with a low fibre content, a practice that results in poor structure of the feed and of the excreta. Poultry, however, require a certain amount of fibre for optimal development of the GIT (Mateos et al., 2012; Röhde et al., 2014; Jiménez-Moreno et al., 2019). Insoluble DF increases gizzard size, nutrient digestibility and GIT health, and reduces gizzard pH (Hetland et al., 2003; Jiménez-Moreno et al., 2016, 2019). A well-developed gizzard is associated with strong contractions of the muscular layers, which ensures the complete grinding of the feed and helps to regulate the flow of the digesta from the gizzard to the small intestine, facilitating the mixing of the chyme and the gastric juices. In addition, a more functional GIT increases mucosa wall motility and prevents the adherence of pathogenic bacteria to the mucosa of the intestines, reducing the risk of enteric disorders (Mateos et al., 2012). As a consequence, the inclusion of moderate amounts of insoluble fibre might improve the structure of the excreta and reduce the incidence of wet litter in poultry.

The beneficial effect of fibre on poultry performance is a subject of debate, with contrasting effects among research studies. Fibre effects vary with type of bird and type and level of fibre. In general, moderate amounts of insoluble fibre improve GIT function and nutrient digestibility in young broilers fed low fibre diets (Mateos et al., 2012). In pullets, the effect of additional fibre on nutrient digestibility is more neutral than in broilers of the same age, probably because of the higher fibre content of commercial pullet diets (Guzmán et al., 2016). An increase in DF in pullets from 10 wk of age to the peak of egg production (pre-peak diets), however, might help to develop the GIT, increasing voluntary FI at the time it is more needed by the hen (Saldaña et al., 2015a; Lohman, 2017). In laying hens, the most relevant effect of DF is the reduction in aggressive behaviour with less incidence on cannibalism and mortality (Aerni et al., 2000; Hartini et al., 2002; Albiker et al., 2015) (Table 8).

Table 8 - Influence of the inclusion of additional fibre sources in the diet on performance of Single Comb White Leghorn hens under free range conditions.

	Crude fibre[1]		Probability
	3.5 %	7 %	
Egg rate, %	94.0	94.0	NS
Feed intake (g/d)	122.6	123.7	NS
Egg weight, g	67.2	67.5	0.19
Feed conversion	2.05	2.06	NS
Mortality, %	7.8	5.5	?
Cannibalism, %	1.2	0.4	0.04
Excreta quality	1.4	1.53	NS

[1] Isonutritive diets to 64 wk of age. The high fibre diet contained: oats, SFH, alfalfa and 2.5 % lignocellulose Albiker et al., 2015

Of note is that DF has two contrasting effects on energy intake in the laying hen: a) an increase because of the increased capacity of the GIT and b) a decrease (under cold conditions) because of the protection caused by the extra feather cover. As a result, the effect of the inclusion of moderate amounts of fibre on FI is often of limited importance in healthy birds.

IV. FEATHER PECKING AND CANNIBALISM

Feather pecking and cannibalism are major problems affecting animal welfare and production in alternative systems for egg production (Aerni et al., 2000; van Krimpen et al., 2005; Ruhnke, 2015; Mens et al., 2019; Kaukonen and Valros, 2019). In practice, the aggressive behaviour is accentuated in non-beak-trimmed birds, and increases as the hens move from traditional to enriched cages, to aviaries, to deep litter barns and finally, to free range and organic production systems. The causes are multiple and include genetics and poor management and nutrition practices, especially during the rearing period (Hartini et al., 2002; van Krimpen et al., 2005). Flock uniformity, lack of attention to chicken behaviour, boredom, bright light and excessive density are some of the poor management practices affecting this multifactorial problem. In particular, it is important to avoid feather pecking during the rearing period and keep the hens busy (increase time dedicated to eating, playing and searching) at all times during the egg production cycle.

Feeding strategies to reduce the incidence of the problem include a) decrease the energy content of the diet while maintaining nutrient specifications (keep hens busy), b) increase the insoluble fibre level of the diet (satiation and GIT comfort), c) avoid faulty nutrition (e.g., deficiencies in met + cys, Na and digestible P levels), d) provide coarse particles (grains, grit, straw) in the floor of the barn and e) supply feed as mash rather than as crumbles (Albiker el al., 2015; van Emous and van Krimpen, 2019).

V. MACRO-MINERALS

Macro-mineral feeding affects the liveability of the laying hen and the quality of the eggs at the end of the production cycle (> 70 wk of age). Three points of interest to maintain egg quality and hen performance are a) use of pre-lay diets with a high Ca content, b) formulate diets based on digestible Ca and phosphorus (P) rather than total Ca and available P, and c) evaluate the use of the electrolyte balance (sodium + potassium – chloride ions), taking into consideration the type of salts used to maintain the balance (Safaa et al., 2019). Recent research (Anwar et al., 2017; Angel et al., 2019) has shown that Ca availability varies widely with factors such as origin, particle size and rheological characteristics of the source and P and phytase content of the diet. Studies conducted in our department (García et al., 2019) show clearly the need for using high Ca diet during the pre-peak (> 15 to 16 wk of age) period to improve shell quality at the end of the egg cycle. In this research, we studied the influence of the nutritive value [nitrogen-corrected apparent metabolizable energy (AMEn), standardized ileal digestible lysine (SID Lys) and Ca] of diets fed to pullets from 15 to 26 wk of age, on productive performance and egg quality of brown egg-laying hens from 27 to 62 wk of age. Five feeding strategies were used (Table 9). Three of them differed in the nutrient content of the diet fed from 15 to 18 wk of age: a) a pullet diet (11.3 MJ AMEn, 6.1 g SID Lys and 10 g of Ca/kg), b) a pre-lay diet (11.5 MJ AMEn, 7.8 g SID Lys and 25 g of Ca/kg), and c) a layer diet (11.5 MJ AMEn, 7.8 g SID Lys and 38 g of Ca/kg), respectively. The other 2 strategies (D and E) consisted of feeding the hens from 15 to 18 wk of age a diet low in energy (10.9 MJ AMEn/kg) and high in SID Lys (8.2 g/kg) with a medium or a high level of Ca (25 vs. 38 g/kg, respectively). After 18 wk (diets A, B and C) or 27 wk (diets D and E) of age, all the hens received the C layer diet to 62 wk of age. Each treatment was replicated 18 times (a cage with 10 hens). Feeding strategy did not affect any of the productive performance traits studied. Cumulatively, all shell quality variables were better ($P < 0.10$) in hens fed the 38 g Ca/kg during the experimental period than in hens fed 25 g Ca/kg or less.

Table 9 - Influence of the level of calcium of the pre-peak diet (15-26 wk)[1] on shell quality of the eggs.

	Pullet[2]	Pre-layer[2]	Layer[2]	Layer plus[3]	Pre-layer plus[3]		
Calcium, g/kg	10	25	38	38	25		
AME_n, MJ/kg	11.3	11.5	11.5	10.9	10.9	Contrasts[4]	
SID Lys, g/kg	6.1	7.8	7.8	8.2	8.2	1	2
Shell strength, kg/cm^2	4.28	4.22	4.29	4.31	4.25	0.684	0.079
Shell weight, g	6.20	6.20	6.29	6.26	6.21	0.304	0.342
Shell weight, % EW	9.97	9.94	10.1	10.1	10.0	0.082	0.039
Shell thickness, µm	389	391	394	395	390	0.135	0.025
Undergrades[5]	1.78	1.60	1.01	0.91	1.46	0.058	0.018
Broken eggs[5]	1.10	0.746	0.600	0.627	0.835	0.015	0.144
Egg-shell less[5]	0.678	0.858	0.414	0.341	0.624	0.399	0.007

[1] From 27 to 62 wk of age all the hens received a common diet with 11.5 MJ, 7.8 g SID Lys and 38 g Ca/kg
[2] Fed from 16 to 18 wk of age.
[3] Fed from 16 to 26 wk of age
[4] 1= Contrast (pullet diets vs. others); 2 = Calcium level effect (2.5 % vs. 3.8 %).
[5] Incidence per each 100 eggs
16 replicates (10 hens each) per treatment

Finally, recent research shows that the requirements for digestible P are very low (< 0.28 % digestible P) in old hens and that an excess at this age reduces egg shell quality. Finally, the formula used to evaluate the electrolyte balance of a feed should be more precise, using laboratory values for the mineral content of the ingredients, and including the $SO_4^=$ ion as a part of the equation. In summary, management practices and sound nutritional strategies are key issues to successful egg production in hen alternative systems.

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IMPACTS OF REARING ENRICHMENTS ON RANGING IN FREE-RANGE LAYING HENS

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Free-range layer systems are increasing globally but range use can be low, particularly when birds are first provided outdoor access. It is recommended to match the rearing system with the layer system for optimal bird welfare (Janczak and Riber, 2015). However, within Australia, pullets destined for free-range systems are typically reared indoors which may hinder their adaptability to the outdoor environment as adults. Rearing enrichments may optimise behavioural development and better prepare hens for outdoor access. This study assessed whether different rearing environments up to 16 weeks of age would affect the initial range use of free-range hens.

Hy-Line Brown chicks (n = 1386) were reared in nine pens with three replicates of three treatments – control (standard litter), novelty (novel objects changed weekly), or structural (perching structures). At 16 weeks pullets were transferred to nine identical pens in an experimental free-range facility with standard floor litter housing, perches, nest boxes, but no additional enrichments. All birds were leg-banded with microchips and radio-frequency identification systems (RFID) were placed within each pen's pop hole. Daily range access was provided from 25 weeks and time spent outdoors was tracked for 5 weeks. The RFID systems had double sensor sets to determine direction of hen travel (out to the range or back inside) and any unpaired (false) readings were filtered out before analysis. Data from the RFID systems provided the average daily time outdoors per individual hen. Video recordings of hens on the range were observed to count the number of hens on the range simultaneously at different distances from the shed across the length of the range area (1.2 to 5m, 5 to 10m, 10 to 20m, and 20 to 31m) every 30 minutes for the first 2 weeks of range access.

General linear models showed no significant differences between rearing treatment groups in the average daily time that individual hens spent outside during the first 2 weeks ($P = 0.34$) but more structural hens were on the range together simultaneously as shown by the counts of hens at specific time points from the video footage ($P < 0.0001$). The structural birds also spent more time outside during weeks 3-5 of range access than the other two treatment groups (mean daily hours ± SEM; structural: 1.71 ± 0.09, novelty: 1.38 ± 0.07, control: 1.40 ± 0.08) $P < 0.0001$). Thus, enrichments during rearing can modify adult hen behaviour and may provide management options for improving bird use of the outdoor range area during the initial access period. Perching structures during rearing may have improved the spatial navigation abilities of those hens (Norman et al., 2019). Further analyses of individual range use across the full flock cycle will show whether any initial differences persist or change with increasing hen age.

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DROUGHT IMPACTS ON PLANT GROUND COVER ON A FREE RANGE EGG FARM

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Summary

Plant ground cover changes were studied on the range of a free range layer flock located in the Mediterranean zone of Australia. Drought conditions were experienced during the study and high-lighted the difficulty for farms in maintaining green ground cover during adverse dry conditions. Hen activity stocked at 1500 birds/ha significantly influenced percentage ground cover, pasture height and botanical composition at flock age 32 weeks compared with 65 weeks. Significant effects were also shown for distance from the shed for ground cover, pasture height and botanical composition. Furthermore, the interaction of flock age x distance from the shed was highly significant for the percentage lucerne (*Medicago sativa*) and lagoon saltbush (*Atriplex suberecta*) growing on the range.

I. INTRODUCTION

Maintaining vegetation on free range farms with fixed ranges is a significant issue (Singh et al., 2017). Nonetheless, free range accreditation programs stipulate palatable vegetation to be available on the range at all times (RSPCA 2015). This is more problematic to achieve during dry seasonal conditions and drought, which are common features of the Australian climate.

II. METHOD

The plant ground cover of a free range farm located in the temperate (Mediterranean) climatic zone of Australia was studied during the drought year of 2018. The long-term average annual rainfall for the area is 470 mm (winter dominant rainfall pattern); however, during 2018, the locality received only 250 mm. Lucerne (*Medicago sativa*) is the main plant sown on the ranges of the farm. The shed (15 x 50 m) and range area (3.8 ha) of the case study flock was established in 2014 and had four previous flocks. Subdivided range areas allow for rotation and resting of pasture every 12 months. Flock size was 5000 Hy-line Brown hens (infra-red beak treated at hatchery) stocked at 1500/ha. Pasture measurements of percentage green ground cover (visual score 0 to 100%), pasture height (cm) and botanical composition (dry weight rank method converted to percentage, Mannetje and Haydock 1963) were made at 10 m, 20 m and 40 m from the shed along four transects (2 transects each side of shed). At each distance (10, 20 and 40 m), 10 quadrats (50 x 50 cm) were assessed. Hens were 32 and 65 weeks of age at the time of pasture measurements (May 2018 and December 2018, respectively). Square root transformation was used for percentage data and Log_{10} for pasture height data. Results were analysed using ANOVA with flock age and distance from the shed as main factors and the interaction flock age x distance.

III. RESULTS

Distance from the shed significantly affected all variates measured (Table 1). Ground cover at 10 and 20 m was similar and increased significantly at 40 m. Pasture height was taller moving further away from the shed. Lagoon saltbush was most commonly found closest to the shed, whereas the opposite was shown for lucerne. Weeds (mostly wire weed – *Polygonum aviculare*) grew predominantly at 10 and 20 m from the shed. Age of flock also significantly

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affected all variates (Table 2). Percentage ground cover, pasture height and percentage lagoon saltbush had all reduced when the flock was 65 weeks old. The reverse occurred for percentage lucerne and weeds, with proportionally more lucerne and weeds at flock age 65 weeks.

Table 1 – Main factor effect of distance from the shed (10, 20 and 40 m) on % ground cover, pasture height, % lagoon saltbush, % Lucerne and % weeds on an egg farm in southern Australia during 2018.

Distance from shed (m)	Ground cover (%)	Pasture height (cm)	Lagoon saltbush (%)	Lucerne (%)	Weeds (%)
10	9.6 (3.10)	5.0 (0.70)	93.1 (9.65)	0.7 (0.83)	1.8 (1.34)
20	9.8 (3.13)	6.9 (0.84)	16.2 (4.03)	42.9 (6.55)	0.9 (0.95)
40	17.8 (4.22)	18.9 (1.28)	0.5 (0.71)	100.0 (10.00)	0.5 (0.71)
LSD $_{5\%}$	0.32	0.06	0.70	0.33	0.37
P value	< 0.001	< 0.001	< 0.001	< 0.001	0.003

Square root transformation was used for % data and Log_{10} for pasture height data. Back-transformed means shown in table with corresponding transformed means shown in brackets. The value for the $LSD_{5\%}$ is based on the transformed data.

Table 2 – Main factor effect of flock age (32 weeks = May 2018 and 65 weeks = December 2018) on % ground cover, pasture height, % lagoon saltbush, % lucerne and % weeds on an egg farm in southern Australia during 2018.

Flock age (weeks)	Ground cover (%)	Pasture height (cm)	Lagoon saltbush (%)	Lucerne (%)	Weeds (%)
32	14.8 (3.84)	12.3 (1.09)	32.1 (5.67)	24.0 (4.90)	0.6 (0.80)
65	9.8 (3.12)	6.1 (0.78)	15.4 (3.92)	45.0 (6.71)	1.43 (1.20)
LSD $_{5\%}$	0.26	0.05	0.54	0.27	0.30
P value	< 0.001	< 0.001	< 0.001	< 0.001	0.010

Square root transformation was used for % data and Log_{10} for pasture height data. Back-transformed means shown in table with corresponding transformed means shown in brackets. The value for the $LSD_{5\%}$ is based on the transformed data.

The only interactions of flock age x distance were for percentage lagoon saltbush and lucerne (Table 3). When the flock age was 32 weeks, lagoon saltbush was common at 20 m. By flock age 65 weeks, the proportion of lagoon saltbush at 20 m had significantly reduced. Conversely, the proportion of lucerne had increased at 20 m. Lucerne remained dominant at 40 m from the shed at both flock ages. The range area reported in this paper will be examined during a second year (rest phase in 2019) and the range area rested in 2018 will be assessed with the new flock during 2019.

Table 3 – Interaction effect of flock age x distance from the shed on % lagoon saltbush and % lucerne on an egg farm in southern Australia during 2018.

	Lagoon saltbush (%)		Lucerne (%)	
Distance from shed (m)	Flock age		Flock age	
	32 weeks	65 weeks	32 weeks	65 weeks
10	95.6 (9.78)	90.6 (9.52)	0.6 (0.77)	0.8 (0.90)
20	42.6 (6.53)	2.3 (1.53)	15.3 (3.91)	84.6 (9.20)
40	0.5 (0.71)	0.5 (0.71)	100.0 (10.00)	100.0 (10.00)
LSD $_{5\%}$	0.94		0.47	
P value	0.001		< 0.001	

Square root transformation was used for % data. Back-transformed means shown in table with corresponding transformed means shown in brackets. The value for the LSD $_{5\%}$ is based on the transformed data.

IV. CONCLUSION

Hen activity at 1500 hens/ha resulted in plant species variations, ground cover decreases and pasture height reduction across the range. Green ground cover was not extensive during the drought with lucerne and lagoon saltbush providing the majority of green cover. Hens had to travel further for green pick as early summer progressed, with lucerne still available at 40 m. Under drought conditions, the provision of green palatable vegetation available on the range at all times would be difficult to maintain at high levels of ground coverage.

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CONSEQUENCES OF OUTDOOR RANGING ON EXTERNAL AND INTERNAL HEALTH PARAMETERS OF HENS FROM DIFFERENT REARING ENRICHMENTS

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Free-range layer pullets are typically reared indoors within Australia, but adult layers go outdoors which might cause poorer adaptation due to the mismatch between rearing and laying environments. Indoor enrichments may optimise physical development of pullets and subsequent welfare as adult free-range hens (Campbell et al., 2019). In the outdoor environment, hens may have greater opportunities for exercise and natural behaviours which might contribute to improved physical health and welfare (Rodriguez-Aurrekoetxea & Estevez, 2016). However, not all hens show equal use of the outdoor range. The objectives of this study were to assess whether adult hens varied in their external and internal health dependent both on their different rearing environments and subsequent variation in range use.

Hy-Line Brown® chicks (n = 1386) were reared indoors for the duration of 16 weeks with 3 enrichment treatments including a control group having no extra materials over floor litter, feed, and water, a novelty group providing novel objects (e.g. balls, bottles, bricks, brooms, brushes, buckets, containers, pet toys, plastic pipes) that changed weekly, and a structural group with H-shaped metal perching structures. At 16 weeks of age the pullets were moved to a free-range system and housed in 9 identical pens with three replicates of each rearing treatment. All hens were leg-banded with microchips and daily ranging was assessed from 25 to 64 weeks via radio-frequency identification technology. At 64-65 weeks of age, a total of 308 hens (statistical unit) across all rearing treatments and pen replicates were selected based on their range use patterns: no outdoor access, low daily outdoor access (1.4h or less daily), and high daily outdoor access (5.2 - 9 h daily). The external health and welfare parameters were evaluated via assessment of plumage condition, toenail length, pecking wounds, and body weight, and internal parameters via post-mortem assessment of internal organ weight and keel bone damage including whole-body CT scanning for body composition.

General Linear Mixed Models showed the control hens had the lowest feather coverage ($P < 0.0001$) and a higher number of comb wounds ($P = 0.03$) than both the enriched groups. The high outdoor rangers had fewer comb wounds ($P = 0.04$), shorter toenails ($P < 0.0001$) and the highest feather coverage ($P < 0.0001$), but lower body weight ($P < 0.0001$) than the indoor hens. The enrichment treatments did not affect the muscle, fat, and bone composition of hens ($P > 0.05$), but high outdoor ranging decreased both body fat and muscle composition (both $P < 0.0001$). The novelty group had lower spleen weights than the control hens ($P = 0.009$) but neither group differed from the structural hens. The high outdoor hens showed the highest spleen ($P = 0.01$) and empty gizzard weights ($P = 0.04$). Both the rearing enrichments and ranging had no effect on overall keel bone damages (all $P \geq 0.19$). Rearing treatments affected hen health and welfare at the later stage of the laying cycle but variation in ranging had a greater impact. Rearing enrichments thus might be recommended for positive effects on hen welfare but management of outdoor access is important in free-range systems.

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A NOVEL TEST FOR POULTRY WELFARE

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Summary

Welfare is of prime concern in the Australian Egg Industry and is highlighted with the ever-increasing demand for free range eggs. There have been huge changes in the egg industry in recent times to accommodate laying hens' welfare, but what is driving this change? Some argue it is supermarket monopolies; others suggest it is people's perceptions of hen welfare. Here we present a new scientific biomarker test that can used to determine the welfare and stress level of laying hens. The preliminary research presented in this report has surveyed cage, barn and free-range management systems to determine their welfare/stress state. This test has the potential to be used as an auditing tool for future investigations into the welfare/stress of laying hens.

I. INTRODUCTION

Heavily driven by public perception and large supermarket monopolies, welfare is of major concern for the poultry industry. The past few decades have seen development of poultry practices with a heavy focus on the welfare of poultry. There have been huge changes implemented to accommodate improvements in welfare, particularly in the layer industry. There are now a number of production systems including caged and free range which provide the consumer a conscious choice on how their eggs have been produced. These advances are closely regulated in most countries with regular inspections and audits to ensure the industry is committed to improving welfare standards. While these developments have undoubtedly facilitated an increase in perceived welfare standards, there is little research to confirm that there has been an actual increase in welfare standards and reduced stress on the birds. Many of the demands placed on the industry in terms of welfare are essentially based on public perception and interpretation, thus making it difficult to objectively assess the real welfare situation. Currently, there are a number of behavioural and other tests (cortisone levels) that have provided an insight into the welfare of poultry, but to date there is no clear scientific test that can be attributed to welfare.

Here we discuss a clear-cut test that will determine the welfare/stress on layers in various production systems. This will enable the industry to provide accurate scientific information on the welfare status of their production systems. This test will also enable auditors to directly test welfare during the course of their routine inspections. Over the last 30 years, a dramatic increase in society's interest in the welfare of farm animals has arisen (Fraser, 2001; Coleman, 2008) and consequently there has been increasing scrutiny of the use of farm animals. A current weakness in studying animal welfare is that there are differing definitions of animal welfare (Fraser 2003; Sandøe et al., 2004). Together with a limited number of evidence-based assessments of welfare, there is need to develop further scientific quantitative assessments to allow producers and the industry to make decisions about the improvement of welfare in these systems. A commonly used biomarker of stress in avian species is measurement of corticosterone in blood. Corticosterone is the major adrenal glucocorticoid hormone that increases in birds under conditions of stress. Corticosterone has short-term effects on the physiology and behaviour of laying birds and also on their long-term performance. A wide variety of stressors, including environmental, temperature and humidity, housing space, feed

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and water restrictions and transport conditions, increase serum corticosterone concentrations in poultry.

Unfortunately, there are major practical difficulties with the measurement of blood concentrations of corticosterone as a biomarker of stress responses, due to the fact that the act of sampling serum from birds can have a profound effect on corticosterone levels as early as 45 seconds after restraint (Beuving and Vonder, 1978). Non-invasive techniques have been developed looking at the corticosterone in egg yolk and albumen (Singh et al., 2009; Royo et al., 2008) and faecal droppings (Rettenbacher and Palme, 2009; Rettenbacher et al., 2004). These techniques have reduced the sampling stress on the birds but can be time consuming and measure total corticosterone levels which does not distinguish between free (biologically active) and bound (not biologically active) corticosterone (Hemsworth and Coleman, 2011). Recently, a class of small non-coding RNAs, namely microRNAs (miRNA), that regulate gene expression and have a critical role in many biological and pathological processes, have been discovered. Studies investigating diseases in humans and other animals have shown clear differences in the expression patterns of miRNAs in serum from healthy compared to disease states (Chen et al.; Liu et al., 2011; Zhao et al., 2010; Gilad et al., 2008; Zhou and Verne, 2011; Schrauder et al., 2012). These studies suggest that the profile patterns of serum miRNAs are useful as biomarkers in a range of conditions, including welfare status. Serum miRNAs are packaged in exosomes and these encapsulated miRNAs have been found also in human breast milk (Zhou et al., 2012; Kosaka et al., 2010) and bovine milk (Chen et al., 2010; Hata et al., 2010). This paper will explore the use of these robust biomarkers as a means to identify stress in laying hens.

II. THE CURRENT STUDY

Here we present the first miRNA based stress test for laying hens. We believe that this test is ready for testing at the industry level. This test has the capacity to identify if hens are stressed at the flock level by using pooled samples and thus would provide real benefit for assessing stress in a range of farming and management systems. This test has the potential to play a role in the auditing of the stress/welfare of chickens across Australia.

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A PHARMACOLOGICAL INTERVENTION MODEL TO ASSESS POSITIVE AFFECTIVE STATES IN LAYING HENS

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It is widely accepted that the absence of suffering no longer defines animal welfare and that positive affective experiences are important (Mellor 2015). However, there are few valid and reliable tools available to comprehensively assess positive affective states of hens, particularly practically on farm. Pharmacological interventions that disrupt specific neural pathways have been shown to be a useful tool when validating indicators of affective states and can assist to manipulate affective states in order to investigate novel indicators such as biomarkers associated with affective states (Nasr et al. 2013). With this approach, we aimed to validate a pharmacological intervention to block the subjective hedonistic phase associated with reward in laying hens via the administration of the opioid antagonist, nalmafene. We predicted that hens that did not experience a positive affective state would show minimal anticipatory behaviour when the same reward was later presented.

Eighty Isa brown hens at 80-85 weeks of age were randomly allocated to either a control (C) or nalmafene (N) treatment group. On day 1 through to 4, hens were dosed intramuscularly into the pectoral muscle with 0.9% saline (C) or nalmafene dissolved in saline (N) at a dose rate of 0.4mg/kg derived from Savory et al (1989). Exactly 30 minutes after dosing, hens were presented with live mealworms in a transparent closed food container at front of their cage. Hens could see the mealworms and could reach the container but could not access the mealworms. After two minutes, the lid was opened and hens were provided with access to the meal worms for five minutes. Latency to peck the container and number of pecks when the lid was closed was calculated as an indicator of anticipation. Kaplan-Meier survival (latency and censored data), generalized linear models (pecks) or Mann-Whitney comparisons (behavior) with treatment, day and the interaction where appropriate.

More hens from the control group (40-100%) pecked the closed mealworm container and were quicker to do so than hens from the nalmefene treatment group (10 - 40%) on all days (day 1: C - 95.1 ± 8.8 s, N - 108.1 ± 8.3 s, $\square^2_{(39,1)} = 4.3$, P = 0.038; day 3: C - 42.3 ± 11.4 s, N - 110.1 ± 7.1 s, $\square^2_{(39,1)} = 17.15$, P < 0.001; day 4: C - 18.4 ± 7.9 s, N - 104.1 ± 8.7 s, $\square^2_{(39,1)} = 29.8$, P < 0.001). The average number of pecks on the closed mealworm container increased over time by hens in the control group (interaction between day and treatment: $\square^2_{(98,1)} = 7.7$, P = 0.005). Treatment hens pecked fewer times than the control hens at all-time points ($\square^2_{(98,1)} = 408.7$, P < 0.001). Behavioural analysis showed no indication that nalmefene treated hens were sedated, nauseous or fearful (immobility, resting, preening and alert all P > 0.05).

We provide evidence that nalmafene may be an effective pharmacological intervention to block the positive affective reward state in laying hens and may be utilised to develop science-based measures to identify novel biomarkers of positive affective state.

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PERCHES - ENVIRONMENTAL ENRICHMENT OR MECHANICAL CHALLENGE?

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Bacterial chondronecrosis with osteomyelitis (BCO) is an infective leg condition that results in lameness, affecting meat chickens internationally. BCO can be induced using mechanical challenges such as wire ramps (Wideman 2016). Based on a study wherein perches had a negative impact on bird latency to lie (LTL) at 42 days (d) old (Phibbs et al. 2020) perches may be a mechanical challenge to birds also, reducing their leg health in a similar way to Wideman's ramps. This study compared the impact of wire ramps and perches on meat chicken mobility and the incidence of detached femoral caps (DFC), which can be indicative of BCO.

Day old Cobb-500 chicks were randomly allocated to one of three treatments: control, perch or wire ramp. Each treatment had six replicate pens, with 42 birds/pen (28kg/m^2 stocking density at 42 d). One perch or ramp ran down the length of the pen between the feeders and drinkers, requiring the birds to pass over them to access food/water. Each wooden perch was 4.2 cm wide and 10 cm off the ground. Wire ramps were 10 cm high and 30cm across, creating a 30° angle from the ground on both sides. Perches and ramps were added to pens at 7 d and remained throughout the 42 d study. Overall feed consumption and weight gain were measured. Behavioural observations were made at five consistent time points every second day wherein all birds in the pen were categorised as either drinking, feeding, active or resting. Birds interacting with the ramps/perches were counted as either actively interacting (AI) when climbing/standing on them or passively interacting (PI) when perching. At 35 d, eight visually male birds were selected from each pen to undergo LTL, and then scored for hock burn (HB) and footpad dermatitis (FPD). At 42 d, seven different male birds were selected to undergo the same assessments, after which they were euthanased and assessed for DFC and FHN. Analysis to determine impact of treatment was performed using IBM SPSS Statistics version 24.

Perch and ramp use peaked at week 3, declining thereafter. Birds with access to the ramps were significantly more active than control birds ($P = 0.023$) and the ramps induced significantly more AI and PI than the perches ($P < 0.001$). Other activity categories were not affected. There was no effect of treatment on bird weight, FCR or LTL. When analysed against treatment, leg health observations were all insignificant except for HB at 35 d ($P = 0.002$) and FPD at 42 d ($P = 0.002$), which were both significantly more prevalent in birds with access to ramps. Perches were associated with a higher prevalence of DFC at 42 d ($P = 0.055$).

There was some evidence that ramps had a negative impact on leg health, but not for observations typical of BCO, such as prevalence of DFC or reduced mobility. Higher prevalence of DFC in birds with access to perches gives some weight to the theory that perches may present a mechanical challenge to birds; however this is not clear-cut. In this study, perches and ramps differed in their effect on leg health and the impact of perches overall remains inconsistent across the literature (Groves & Muir 2013; Phibbs et al. 2019, 2020).

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TIBIA QUALITY, TISSUE MINERALIZATION AND LIVER LIPID PEROXIDATION OF BROILERS IN RESPONSE TO DIFFERENT SOURCES AND LEVELS OF COPPER

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Copper (Cu) is a vital element involved in various physiological and biochemical processes, namely cellular metabolism and enzyme systems. Higher levels of copper than nutritional requirements (up to 250 mg/kg) are supplemented to poultry diets. However, if in excess, excreted Cu can contaminate the environment. High levels of copper sulphate ($CuSO_4$) may damage other dietary nutrients through increased oxidation. Meanwhile, copper hydroxychloride (IBC) is less soluble than $CuSO_4$ at neutral pH; therefore it is less destructive and increases stability of essential nutrients such as vitamins and fats in the feed and within the bird (Luo et al., 2005).

This study aimed to examine the efficacy of $CuSO_4$ compared with that of IBC on tibia breaking strength, ash and Cu content, Cu concentration of distal ileal digesta and liver malondialdehyde (MDA) concentration in broiler chickens. A total of 864 Ross 308 male day-old chicks (41.9 ± 0.19 g) were fed wheat-soybean meal based starter diets from day 1-14 and the grower diets from day 14-35. Birds had *ad libitum* access to feed and water throughout the study period. There were eight dietary treatments replicated six times in 48 floor pens: negative control (NC) treatment with no supplemental Cu, 15 and 200 mg/kg Cu from $CuSO_4$ or 15, 50, 100, 150 and 200 mg/kg Cu from IBC. On d 35, the right tibia, liver and ileal digesta samples were collected from three representative birds per replicate after euthanasia. The tibias were subjected to breaking strength by an Instron instrument. Mineral concentration in liver and ileal digesta were determined by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) technique. MDA level in the liver was determined using a lipid peroxidation (MDA) assay kit (AB118970, abcam®, UK).

Tibia strength increased with the increments of supplemental Cu. The highest tibia breaking strength value was observed in birds given the 200 mg/kg IBC diet (338 N/mm^2), suggesting higher bioavailability of Cu from the hydroxy source. The lowest tended to be seen in birds fed diets without supplemental Cu (285 N/mm^2, $P = 0.059$). Dietary treatment had no significant effect on tibia ash content (average of 45.5 %, $P > 0.05$) or tibia Cu content (average of 1.94 µg/g, $P > 0.05$). Liver Cu level was greater in birds fed the 200 mg/kg $CuSO_4$ diet (18.9 µg/g, $P < 0.01$) and 200 mg/kg IBC diet (14.6 µg/g) compared to those on any other treatment. The Cu composition of ileal digesta increased with increasing of supplemental Cu in the diets ($P < 0.01$). The highest ileal digesta Cu level was observed in birds fed $CuSO_4$ at 200 mg/kg (995 µg/g), indicating that more Cu from $CuSO_4$ was unavailable and excreted. The concentration of liver MDA was not significantly affected by the dietary treatments (average of 0.801 nmol/mg, $P > 0.05$), indicating that high dietary Cu did not negatively impact hepatic oxidation.

It can be concluded that supplementation of copper from IBC is beneficial to broiler chickens in promoting bone development. The inclusion of 200 mg/kg IBC resulted in the highest bone strength and similar organ Cu accumulation to 200 mg/kg $CuSO_4$.

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BARLEY PARTICLE SIZE AND SUPPLEMENTAL ENZYMES: INFLUENCE ON GROWTH PERFORMANCE AND NUTRIENT UTILISATION OF BROILER STARTERS

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Summary

The influence of barley particle size and enzyme supplementation on performance and nutrient utilisation of broiler starters fed pelleted barley-based diets was evaluated in a 21-d experiment. Two barley particle sizes (fine and coarse) and four methods of enzyme supplementation (non-supplemented [control], carbohydrase [Carb], phytase [Phy] and combination of carbohydrase and phytase [Carb + Phy]) were evaluated in a 2 × 4 factorial arrangement of eight treatments. Regardless of barley particle size, Carb + Phy tended ($P = 0.056$) to increase weight gain compared to the diet with no enzyme. The response of feed intake to supplemental enzymes interacted ($P < 0.05$) with barley particle size, with Phy increasing feed intake only in fine barley diets. Both coarse particle size and supplemental carbohydrase, individually or in combination with Phy, reduced ($P < 0.001$) feed per gain. Greater digestibility of dry matter, nitrogen and fat was observed ($P < 0.01$ to 0.05) in birds fed coarse barley diets. Dry matter digestibility was improved ($P < 0.05$) by all enzyme treatments. Carbohydrase supplementation, individually or in combination with Phy, increased ($P < 0.05$) starch and fat digestibility coefficients. Results from this study showed that feeding coarse barley particles and supplementation of Carb in pelleted barley-based diets is beneficial in terms of feed efficiency and nutrient utilisation.

I. INTRODUCTION

Cereal grains are ground prior to feed mixing to reduce the particle size with the aim of modifying their physical characteristics. This facilitates handling, mixing and further processing (extrusion and pelleting) and increases the exposure of nutrients in the endosperm to digestive enzymes (Amerah et al., 2011). Available recommendations regarding optimum particle size are contradictory, due to the confounding effects from several factors including grain type, feed form, complexity of the diet, endosperm hardness, grinding method and particle size distribution (Amerah et al., 2007a). The influence of grain particle size on growth performance and nutrient utilisation of poultry fed maize- (Amerah and Ravindran, 2009; Naderinejad et al., 2016) and wheat- (Amerah et al., 2007b; Abdollahi et al., 2019) based diets has been examined, but corresponding studies with barley are lacking.

In addition to the commonly used carbohydrases that target non-starch polysaccharides, phytases are usually added to cereal-based diets to facilitate the release of phytate-bound phosphorus and to minimise the phosphorus effluent from intensive poultry production (Ravindran et al., 1995). Several researchers (Ravindran et al., 1999; Wu et al., 2004) have evaluated the individual and combined supplementation of carbohydrases and phytases to cereal-based diets and reported inconsistent effects requiring further elucidation. Moreover, only a limited number of studies were conducted to evaluate the interactions between different particle sizes of maize (Kasim and Edwards, 2000; Amerah and Ravindran, 2009) and wheat (Amerah et al., 2008) and supplemental enzymes. However, the findings from these studies are

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contradictory and, to the authors' knowledge, corresponding studies with barley are not available. Accordingly, the present study was conducted to assess the potential interactive influence of barley particle size and carbohydrase and phytase addition, individually or in combination, on growth performance and nutrient digestibility of broiler starters offered pelleted diets.

II. MATERIALS AND METHODS

A hulled barley cultivar (Fortitude; Amylopectin, 343 g/kg; Amylose, 267 g/kg; Perera et al., 2019) was ground in a hammer mill to pass through 2.0 and 8.0 mm screens, to achieve fine and coarse barley particles, respectively. Nutrient composition, nitrogen (N)-corrected apparent metabolisable energy and standardised digestible amino acid contents of barley, determined in a previous study (Perera et al., 2019), were used in formulating a basal diet that contained an adequate concentration of non-phytate phosphorus (4.8 g/kg). Two diets, mixed using fine or coarse barley, were developed into eight dietary treatments using a multi-component non-starch polysaccharide-degrading enzyme (Ronozyme® Multigrain) and a phytase (Ronozyme® HiPhos). Four methods of enzyme supplementation were employed: non-supplemented (control), carbohydrase (0.15 g/kg of feed; Carb), phytase (0.10 g/kg; Phy) and combination of carbohydrase and phytase (0.15 and 0.10 g/kg, respectively; Carb + Phy) were used in this study. The average analysed activities of phytase, endo-1,3 (4)-β-glucanase and endo-1,4-β-xylanase from enzyme-supplemented diets were 775, 80.5 and 212 IU/kg, respectively. The diets contained 5.0 g/kg of titanium dioxide as an indigestible marker. Each of the eight dietary treatments was offered *ad libitum* to six replicate cages (eight birds per cage). Body weights and feed intake were recorded at weekly intervals throughout the 21-d trial. On d 21, ileal digesta were collected for determination of the coefficient of apparent ileal digestibility (CAID) of dry matter (DM), N, starch and fat.

III. RESULTS AND DISCUSSION

Enzyme supplementation tended ($P = 0.056$) to increase the weight gain of birds with a synergetic effect from the combined use of enzymes (Table 1). A significant ($P < 0.05$) barley particle size × enzyme interaction was observed as Phy increased feed intake only in fine barley diets, partly due to the poor nutrient digestibility of birds fed fine barley diets. Both coarse particle size and supplemental carbohydrase, either individually or in combination with phytase, reduced feed per gain (F/G; $P < 0.001$). Coarse particles reduced F/G by 2.1 points, whilst Carb and Carb + Phy reduced the F/G by 2.9 and 2.1 points, respectively. The improvement in F/G due to coarse grinding in the current study is in contrast to previous research (Amerah et al., 2007b; Chewning et al., 2012) reporting no effect of grain particle size on performance of birds fed pelleted diets. Based on the lack of effect from grain particle size in pelleted diets, previous studies hypothesised that pelleting can mask the influence of particle size. However, the present results suggest that the effects of feed particle size on performance might be maintained even after pelleting.

The main effects of barley particle size and supplemental enzyme on nutrient digestibility are summarised in Table 2, as there was no significant interaction between particle size and enzyme. The improvements ($P < 0.01$ to 0.05) of 3.1, 3.2 and 4.3% in CAID of DM, N and fat, respectively, in coarsely-ground barley diets are contrary to the findings of Naderinejad et al. (2016) and Abdollahi et al. (2019), who reported no effect of maize and wheat particle size, respectively, on ileal digestibility of DM, N and fat. Well-developed gizzards in birds fed coarse barley diets (data not reported) might have contributed to the improved digestibility of DM, N and fat through extensive grinding, mixing and lower pH (Svihus, 2011). Moreover, coarse particles can reduce the digesta passage rate through the

gizzard and therefore are retained longer than finer particles in the digestive tract (Amerah et al., 2007a) increasing the exposure time of nutrients to digestive enzymes.

Table 1- The influence of barley particle size and carbohydrase (Carb) and phytase (Phy) supplementation, individually or in combination (Carb + Phy), on weight gain (WG; g/bird), feed intake (FI; g/bird) and feed per gain (F/G; g feed/g gain) of broiler starters[1] (0-21 d).

Particle size[2]	Enzyme	WG	FI	F/G
Fine	No enzyme	1185	1477bc	1.246
	Carb	1198	1442c	1.214
	Phy	1208	1519a	1.256
	Carb + Phy	1223	1501ab	1.235
Coarse	No enzyme	1197	1474bc	1.235
	Carb	1204	1456c	1.209
	Phy	1199	1463c	1.220
	Carb + Phy	1215	1458c	1.203
SEM[3]		9.8	12.6	0.0074
Main effects				
Particle size				
Fine		1203	1485	1.238a
Coarse		1204	1463	1.217b
Enzyme				
	No enzyme	1191	1475	1.240a
	Carb	1201	1449	1.211b
	Phy	1204	1491	1.238a
	Carb + Phy	1219	1479	1.219b
Probabilities, P ≤				
Particle size		0.962	0.018	0.001
Enzyme		0.056	0.014	0.001
Particle size × Enzyme		0.634	0.026	0.107

Means in a column not sharing common letters (a,b,c) are different (P < 0.05).
[1]Each value represents the mean of six replicates (eight birds per replicate).
[2]Fine and coarse barley particles were achieved by grinding whole barley in a hammer mill to pass through 2.0 and 8.0 mm screens, respectively.
[3]Pooled standard error of mean.

Enzyme supplementation, regardless of barley particle size, improved (P < 0.05) the DM, starch and fat digestibility. Carb, Phy and Carb + Phy improved CAID of DM by 5.7, 4.2 and 5.9%, respectively. Moreover, Carb and Carb + Phy enhanced the starch digestibility by 1.5 and 1.8%, respectively and, fat digestibility by 4.7 and 5.7%, respectively. In this study, however, no effect of supplemental enzymes on jejunal digesta viscosity was observed (data not shown). Therefore, the improvement in CAID of nutrients due to supplemental Carb might be attributed to the degradation of endosperm cell walls by added Carb, consequently releasing encapsulated nutrients and allowing better interaction with digestive enzymes (Kim et al., 2005). Phytate in wheat and barley is largely located in the aleurone layer (Eeckhout and De Paepe, 1994). The improvement in CAID of DM due to supplemental Phy, at least in part, was caused by the action in disrupting cell wall and consequent release of encapsulated nutrients in a manner similar to that of Carb (Ravindran et al., 1999). In conclusion, the present study showed that feeding coarse barley particles and supplementation of Carb improved feed efficiency and nutrient utilisation in broiler starters fed pelleted diets. The effect of grain particle size was preserved even after pelleting, as indicated by feed intake, feed efficiency and DM, N and fat digestibility values. The combination of Carb and Phy tended to improve weight gain, but caused no further improvements in nutrient utilisation.

Table 2 - The influence of barley particle size and carbohydrase (Carb) and phytase (Phy) supplementation, individually or in combination (Carb + Phy) on coefficient of apparent ileal digestibility (CAID) of dry matter (DM), nitrogen (N), starch and fat in 21-d old broilers[1].

		CAID			
		DM	N	Starch	Fat
Main effects					
Particle size[2]					
Fine		0.609b	0.744b	0.939	0.837b
Coarse		0.628a	0.768a	0.937	0.873a
Enzyme					
	No enzyme	0.595b	0.741	0.929b	0.831c
	Carb	0.629a	0.763	0.943a	0.870ab
	Phy	0.620a	0.761	0.935ab	0.842bc
	Carb + Phy	0.630a	0.758	0.946a	0.878a
Probabilities, P <					
Particle size		0.022	0.002	0.600	0.003
Enzyme		0.012	0.130	0.044	0.014
Particle size × Enzyme		0.754	0.650	0.877	0.108

Means in a column not sharing common letters (a,b,c) are different (P < 0.05).
[1]Each value represents the mean of six replicates (six birds per replicate).
[2]Fine and coarse barley particles were achieved by grinding whole barley in a hammer mill to pass through 2.0 and 8.0 mm screens, respectively.

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FEED FORM AFFECTS THE APPARENT METABOLISABLE ENERGY OF INDIVIDUAL INGREDIENTS TO DIFFERENT EXTENTS IN BROILER CHICKENS

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Summary

The current study was designed to examine the influence of feed form (FF) on the apparent metabolisable energy (AME) and nitrogen-corrected AME (AMEn) content of four grains (maize, sorghum, wheat and barley) and three protein sources (meat and bone meal [MBM], soybean meal [SBM] and canola meal [CM]) in broiler chickens. The AME was measured, from 25 to 28 d post-hatch, following the direct method (grains) and the difference method (protein sources) by total excreta collection. No interaction ($P > 0.05$) between grain type and FF was found for the AMEn. Maize and sorghum showed the highest ($P < 0.05$) AMEn, barley the lowest and wheat being intermediate. There was a significant ($P < 0.05$) interaction between protein source and FF for the AMEn. Pelleting reduced ($P < 0.05$) the AMEn of MBM, had no effect ($P > 0.05$) on SBM but increased ($P < 0.05$) that of CM. Overall, pelleting increased the AME and AMEn of all four cereal grains, but its effect on protein sources varied depending on the ingredient.

I. INTRODUCTION

Estimating the available energy of feed ingredients is fundamental to formulating a well-balanced diet and lower the production cost. Measurement of apparent metabolisable energy (AME) is the accepted standard procedure for evaluating the available energy value of ingredients for poultry, as it is simple and straightforward and considers most of the energy losses after digestion and metabolism (Carré et al., 2014).

The measurement of AME content of feed ingredients for broilers, regardless of the methods (direct, difference and regression), is normally conducted using mash diets and the impact of feed form (FF; mash vs. pellets) on the AME and nitrogen-corrected AME (AMEn) estimates is largely overlooked. To the authors' knowledge, no study has previously investigated the effect of FF on the AME and AMEn of individual ingredients. However, published data on the effects of FF on AME and AMEn of complete diets in broilers have been equivocal (Svihus et al., 2004; Amerah et al., 2007; Roza et al., 2018). Hussar and Robblee (1962) reported that pelleting had no effect on dietary AME or AMEn in a wheat-based diet. Svihus et al. (2004) reported an increase in AME value of a wheat-based diet as a result of pelleting compared to the same diet in mash form. In contrast, negative effects of pelleting on the AME and AMEn have also been reported in wheat- and sorghum-based diets (Amerah et al., 2007; Abdollahi et al., 2014).

These findings suggest that the estimates determined in assays using mash diets might over- or under-estimate the AME of individual ingredients when used in complete pelleted diets. This calls into question the application of AME values obtained with mash diets to commercial situations where the majority of broiler feeds is pelleted. Therefore, the objective of the present study was to investigate the impact of FF in energy evaluation of individual feed ingredients for broiler chickens.

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II. MATERIALS AND METHODS

The present study was divided into two experiments; the first experimental design was a 4 × 2 factorial arrangement of four cereal grains (maize, sorghum, wheat and barley) and two FF (mash vs. pellets), and the second experimental design was a 3 × 2 factorial arrangement of three protein sources (meat and bone meal [MBM], soybean meal [SBM] and canola meal [CM]) and two FF (mash vs. pellets).

In the first experiment, the AME of test grains were determined using the direct method. Four basal diets were formulated to contain the same inclusion level (962 g/kg) of each grain. In the second experiment, the AME of test protein sources were determined by the difference method. A maize-soybean basal diet was formulated and the test diets, each containing a different protein source, were developed by replacing (w/w) 30% of the basal diet with one of the protein sources. In both experiments, each diet was divided into two equal batches and, one was offered in mash form and the second was pelleted. On d 18, a total of 288 birds were individually weighed and randomly allocated to 48 cages with six replicates per treatment (six birds/cage). Birds were fed the experimental diets from d 21 until 28 d of age, with the first three d serving as an adaptation period. Diets were offered *ad libitum*. For the determination of AME, feed intake and total excreta output for each replicate were recorded over the last four d of the assay. Sub-samples of excreta were lyophilised, ground and analysed for dry matter (DM), gross energy (GE), and nitrogen (N). Appropriate formulas were used for the calculation of AME and AMEn.

III. RESULTS AND DISCUSSION

The influence of grain type and FF on N retention, AME and AMEn for broiler chickens is shown in Table 1. The influence of treatments on the AMEn followed a similar pattern to that of the AME. Neither the AMEn nor the N retention were subject to an interaction ($P > 0.05$) between grain type and FF. However, grain type ($P < 0.001$) and FF ($P < 0.01$) had a significant effect on the AMEn. The AMEn of maize and sorghum were similar ($P > 0.05$) and higher ($P < 0.05$) than those of wheat and barley. Barley showed the lowest ($P < 0.05$) AMEn. Pelleting increased ($P < 0.05$) the AMEn values, regardless of the grain type. Grain type influenced ($P < 0.001$) the N retention, with bird fed maize having higher N retention compared to those fed the other grains.

Table 1 - Influence of grain type and feed form on nitrogen (N) retention, apparent metabolisable energy (AME) and nitrogen-corrected AME (AMEn) (MJ/kg DM) in broilers measured from 25 to 28 d posthatch[1].

Grain type	AME	AMEn	N retention (%)
Main effects			
Grain type			
Maize	15.37[a]	15.11[a]	53.0[a]
Sorghum	15.60[a]	15.32[a]	39.5[b]
Wheat	14.15[b]	13.84[b]	39.1[b]
Barley	13.43[c]	13.10[c]	41.2[b]
Feed form			
Mash	14.51[b]	14.23[b]	41.9
Pellet	14.76[a]	14.45[a]	44.5
Probabilities, P ≤			
Grain type	0.001	0.001	0.001
Feed form	0.009	0.006	0.244
Grain type × Feed form	0.981	0.949	0.698

Means in a column not sharing a common letter (a-c) are significantly different ($P < 0.05$).
[1] Each value represents the mean of six replicates (six birds per replicate).

The increase in AME and AMEn of cereal grains by 0.25 MJ/kg and 0.22 MJ/kg, respectively, due to pelleting in the current study is in agreement with a previous study (Roza et al., 2018), reporting that pelleting increased AME and AMEn values of a maize-based diet by 0.27 and 0.26 MJ/kg, respectively, compared to mash form. In contrast, pelleting was reported to reduce the AME of a wheat-based diet by 0.46 MJ/kg (Abdollahi et al., 2011) and AMEn of a maize-based diet by 0.17 MJ/kg (Abdollahi et al., 2018). Amerah et al. (2007) reported a significant negative effect of pelleting wheat-based diet on AMEn (11.81 MJ/kg) compared to mash diet (12.54 MJ/kg). These contradictory results could be related to differences in diet composition, as the above studies have been conducted with complete feeds.

The lack of FF effect on N retention is in agreement with the study by Selle et al. (2012), who reported no effect of FF on N retention in sorghum-based diets. These findings are also in agreement with those by Woyengo et al. (2010) who found no effects of FF on the N retention of broilers fed a maize-soybean meal-based diet. Similarly, Favero et al. (2012) reported that FF had no influence on the N retention in turkeys fed a maize-soybean meal-based diet. In contrast, other studies showed that pelleting increased the N retention in wheat- (Pirgozliev et al., 2016) and maize-based diets (Zatari and Sell, 1990). These contradictory results could be related to differences in pelleting conditions and diet composition.

The influence of FF on N retention, AME and AMEn of protein sources for broiler chickens is shown in Table 2. Significant ($P < 0.05$) protein source × FF interactions were observed for the AMEn. Pelleting did not have any effect ($P > 0.05$) on the AMEn of SBM, reduced ($P < 0.05$) that of MBM, and increased ($P < 0.05$) the AMEn of CM. Significant ($P < 0.05$) protein source × FF interactions were observed for N retention. Feeding pelleted diets increased ($P < 0.05$) the N retention for MBM, but had no effects on those for SBM and CM.

Table 2 - Influence of protein source and feed form on nitrogen (N) retention, apparent metabolisable energy (AME) and nitrogen-corrected AME (AMEn) (MJ/kg DMs) in broilers measured from 25 to 28 d posthatch[1].

Protein source	Feed form	AME	AMEn	N retention (%)[2]
Meat and bone meal	Mash	17.03^{a}	14.79^{a}	47.2^{d}
	Pellet	16.42^{b}	14.23^{b}	51.4^{bc}
Soybean meal	Mash	11.47^{c}	10.06^{c}	55.9^{a}
	Pellet	11.05^{c}	9.88^{c}	54.2^{ab}
Canola meal	Mash	9.04^{d}	7.87^{e}	51.4^{bc}
	Pellet	9.48^{d}	8.44^{d}	49.9^{cd}
SEM		0.202	0.182	1.16
Main effects				
Protein source				
Meat and bone meal		16.72	14.51	49.3
Soybean meal		11.26	9.97	55.0
Canola meal		9.26	8.16	50.7
Feed form				
Mash		12.51	10.91	51.5
Pellet		12.32	10.85	51.8
Probabilities, P ≤				
Protein source		0.001	0.001	0.001
Feed form		0.245	0.719	0.738
Protein source × Feed form		0.033	0.014	0.024

Means in a column not sharing a common letter (a-e) are significantly different ($P < 0.05$).
[1] Each value represents the mean of six replicates (six birds per replicate).
[2] N retention for the test diets.

Pelleting reduced the AME and AMEn by 3.58 and 3.94%, respectively, only for MBM. However, pelleting increased AMEn of CM by 7.25% compared to the mash form, which may be attributed to the effect of heat and pressure in disrupting the structure of the cell walls, thus releasing the lipids contained in the oil bodies (Jiménez-Moreno et al., 2009). No previous studies have compared the effect of FF on the AME or AMEn of protein sources for broilers. The influence of pelleting on N retention was pronounced only for MBM with an increase of 8.9% compared to the mash form.

To our knowledge, there are no reports available on the effect of FF on AME or AMEn of individual cereal grains or protein sources and the current findings suggest that the application of AME or AMEn values determined based on assays using mash diets might result in over- or under-estimation. Therefore, energy evaluation of individual ingredients under different feed processing conditions is crucial for formulating well-balanced diets for broilers.

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STANDARDISED ILEAL AMINO ACID DIGESTIBILITY OF INGREDIENTS FOR BROILER CHICKENS IS INFLUENCED BY FEED FORM

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Summary

The current study was conducted to determine the influence of feed form (FF) on the standardised ileal digestibility (SID) of nitrogen (N) and amino acids (AA) in different grain types (GT) and protein sources (PS). The study was divided into two experiments. Experiment 1 was designed as a 4 × 2 factorial arrangement including four GT (maize, sorghum, wheat and barley) and two FF (mash vs pelleted). In experiment 2, a 3 × 2 factorial arrangement including three PS (meat and bone meal [MBM], soybean meal [SBM] and canola meal [CM]) in two FF (mash vs pelleted) was used. A nitrogen-free diet (NFD) was also included. The assay diets and the NFD were fed to birds from day 19 to 23 post-hatch and ileal digesta was collected on day 23. In experiment 1, the GT had a significant ($P < 0.05$) effect on the SID of N and AA. Barley had lower N digestibility than the other grains, and average SID of total AA was higher in maize and sorghum than in barley. The FF showed no significant ($P > 0.05$) effect on SID of N and AA except for cysteine and proline, which were higher ($P < 0.05$) in birds fed mash than those fed the pelleted diet. In experiment 2, the SID of N was higher ($P < 0.05$) in SBM followed by MBM and CM. Histidine was the only indispensable AA influenced by FF, and its SID was reduced ($P < 0.05$) by pelleting. Pelleting, however, resulted in reductions ($P < 0.05$) in the SID of all dispensable AA. Present data demonstrates that FF can influence the measurement of AA digestibility, in particular dispensable AA, in protein sources, and should be taken into account in feed evaluation assays.

I. INTRODUCTION

Knowledge on the digestibility of amino acids (AA) in raw materials is crucial for precise feed formulation, and for more efficient and sustainable use of feed resources. The accuracy of excreta-based digestibility measurements for determining N and AA digestibility may be questionable, due to the variable effects of the caecal microflora on dietary protein utilisation and the contribution of microbial proteins to AA excretion in the excreta of birds. In the ileal digestibility assay these confounding issues are avoided. Ileal AA digestibility values can be referred to as either apparent or standardised/true. Standardised ileal digestibility (SID) involves a correction for the inevitable endogenous AA losses from the gastrointestinal tract (Lemme et al., 2004), and SID values are more additive than the apparent values. Application of SID AA in practical feed formulation would benefit poultry production by improving the accuracy of feed formulation, and reducing diet costs and nitrogen pollution (Cowieson et al., 2019).

A large volume of published data (Ravindran et al., 1998; Lemme et al., 2004; Bryden et al., 2009) on AA digestibility of a range of feed ingredients for broilers is now available. These available values of digestible AA for broilers have been determined using mash diets, due to simplicity. However, the majority of feed used in broiler production is fed in pelleted or crumbled forms, and feed processing, texture and conditions associated with the pelleting process, such as temperature and moisture, have a substantial impact on nutrient digestion

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(Abdollahi et al., 2013a). Furthermore, it has been speculated that hydrothermal treatment of feed may enhance protein digestibility due to denaturation and dissociation of protein macro-structure into smaller subunits (Camire, 1991; Ludikhuyse et al., 2003). On the other hand, steam-pelleting of diets might decrease the protein solubility that compromises its availability for the bird (Svihus and Zimonja, 2011). Recent evidence suggests that the nature of the digestibility response to the pelleting process is dependent on the ingredient and the specific nutrient (Abdollahi et al., 2013b). Therefore, the application of AA digestibility data generated with mash diets to practical situations, where feed is in pelleted form, could be questioned. Based on the above, it was hypothesised that broilers fed the same diet but in different feed forms (FF) may show different AA digestibility values. Consequently, the present study was designed to determine the influence of FF (mash vs pelleted) on the SID of nitrogen (N) and AA in different feed ingredients.

II. MATERIALS AND METHODS

The study was divided into two experiments. A completely randomised design in a 4 × 2 factorial arrangement was used in experiment 1, which included four grain types (GT; maize, sorghum, wheat and barley) and two FF (mash vs pelleted). The cereals were obtained from a commercial supplier and ground in a hammer mill to pass through a screen size of 3.0 mm. The eight assay diets contained 938 g/kg cereals as the only source of AA in the diet. Experiment 2 was designed in a 3 × 2 factorial arrangement including three protein sources (PS; meat and bone meal [MBM], soybean meal [SBM] and canola meal [CM]) in mash and pelleted forms. A N-free diet (NFD) was also used to determine the basal endogenous N and AA losses for the calculation of SID values. Titanium dioxide (TiO2; 5 g/kg; Merck KGaA, Darmstadt, Germany) was added to all diets as an indigestible marker. The pelleted assay diets were steam-conditioned at 70 ºC for 30 seconds and pelleted using a pellet mill (Richard Size Limited Engineers, Orbit 15, Kingston-upon-Hull, UK) equipped with a die ring (3-mm holes and 35-mm thickness). Each of the diets was offered *ad libitum* to six replicate cages (eight birds per cage) from d 19 to 23. On day 23, all birds per cage were euthanised by intravenous injection of sodium pentobarbitone solution and digesta were collected from the distal ileum, pooled within a cage and used for determination of SID of N and AA.

III. RESULTS AND DISCUSSION

The influence of GT and FF on the SID of N and AA are shown in Table 1. There was no GT × FF interaction for the SID of N and AA. The main effect of GT on the SID of N was statistically significant ($P < 0.001$), with lower digestibility in barley-based diets compared to any other grain. Maize-based diets showed similar ($P > 0.05$) average SID values for indispensable AA (IAA) to sorghum, but higher ($P < 0.05$) than wheat and barley. Average total amino acid (TAA) digestibility was higher in maize and sorghum compared to barley. The trends of higher AA digestibility in maize followed by sorghum, wheat and barley are in agreement with previous findings (Lemme et al., 2004; Szczurek, 2009). Feeding pelleted diets reduced ($P < 0.05$) the SID of cysteine and proline and tended ($P = 0.063$) to decrease the SID of glutamic acid. Cysteine is the most heat labile of all AA and its digestibility is affected during hydrothermal treatment (Abdollahi et al., 2013a), most probably due to the formation of disulphide bonds (Wall, 1971).

Table 1 - Influence of grain type (GT) and feed form (FF) on the standardised ileal digestibility[1] (%) of nitrogen (N) and amino acids[2] (experiment 1).

				Dispensable AA				
GT	FF	N	IAA	Cys[3]	Glu	Pro	DAA	TAA
Main effects								
GT								
Maize		82.9^{a}	82.8^{a}	85.7^{a}	89.5^{a}	86.4^{b}	84.5^{a}	83.8^{a}
Sorghum		80.8^{a}	80.4^{ab}	78.1^{b}	84.7^{b}	79.7^{c}	80.4^{a}	80.4^{a}
Wheat		79.7^{a}	75.3^{bc}	86.2^{a}	91.4^{a}	91.2^{a}	80.2^{a}	77.8^{ab}
Barley		71.1^{b}	70.4^{c}	81.5^{ab}	80.4^{c}	80.8^{c}	73.7^{b}	72.3^{b}
Pooled SEM		2.11	2.34	1.79	1.27	1.25	1.85	2.09
FF								
	Mash	79.5	77.8	84.9^{a}	87.7	86.1^{a}	80.9	79.5
	Pellet	77.8	76.7	80.7^{b}	85.3	82.9^{b}	78.4	77.7
Pooled SEM		1.49	1.66	1.27	0.89	0.89	1.31	1.48
Probabilities, P								
GT		0.001	0.003	0.009	0.001	0.001	0.002	0.004
FF		0.445	0.637	0.022	0.063	0.017	0.175	0.394
GT× FF		0.953	0.866	0.933	0.697	0.717	0.858	0.863

Means in a column not sharing a common letter (a-c) are significantly different (P < 0.05).
[1]Apparent digestibility values were standardised using the following basal ileal endogenous flow values (g/kg DM intake), determined by the feeding protein-free diet: crude protein, 17.3; Arg, 0.59; His, 0.27; Ile, 0.54; Leu, 0.87; Lys, 0.57; Met, 0.22; Thr, 1.04; Trp, 0.19; Val, 0.72; Ala, 0.65; Asp, 1.15; Cys, 0.42; Glu, 1.42; Gly, 0.68; Pro, 0.83; and Ser, 0.86.
[2]Each value represents the mean of six replicates (eight birds per replicate). [3]Semi-indispensable amino acids for poultry.
AA = amino acids; IAA = Average digestibility of indispensable amino acids; DAA = Average digestibility of dispensable amino acids; TAA = Average digestibility of all amino acids.
Cys = cysteine; Glu = glutamic acid; Pro = proline.

The influence of PS and FF on the SID of N and AA are shown in Table 2. There were no significant interactions between PS and FF observed for the SID of N and AA. The SID of N was significantly (P < 0.05) higher in SBM than all other protein sources, and higher in MBM compared to CM. Average SID of total AA were similar in SBM and MBM, and higher than that in CM. Lemme et al. (2004) previously reported higher SID of protein in SBM (90%) than CM (76%) and MBM (65%) in broilers. Histidine was the only IAA significantly (P < 0.05) influenced by FF; it was reduced by pelleting. Pelleting, however, resulted in a significant reduction in the SID of all individual DAA, average SID of DAA and TAA. As suggested by Goodarzi Boroojeni et al. (2016), the possible effect of hydrothermal treatment on protein denaturation is not necessarily associated with higher protein or AA digestibility. Overconsumption and overload of nutrients in birds fed pelleted diets have been shown to reduce the digestibility of major nutrients (Abdollahi et al., 2013b, 2018). Also, Engberg et al. (2002) observed smaller gizzard and pancreas and lower amylase, lipase, trypsin and chymotrypsin activities in pellet-fed birds than those fed mash feed. Therefore, the lower SID of AA in pelleted diets in the present study might be partly explained by reduced activity of proteolytic enzymes in the digestive tract. In addition, the lower digestive organ weights (relative to feed intake) induced by feeding pelleted diets, might shorten digesta retention time, compromising digestion and absorption of nutrients (Abdollahi et al., 2018). Overall, the current study suggests that FF can influence the measurement of AA digestibility, in particular dispensable AA, in protein sources and, therefore, should be considered in future AA digestibility assays.

Table 2 - Influence of protein source (PS) and feed form (FF) on the standardised ileal digestibility[1] (%) of nitrogen (N) and amino acids[2] (experiment 2).

			Indispensable AA		Dispensable AA								
PS	FF	N	His	IAA	Ala	Asp	Cys[3]	Glu	Gly[3]	Pro	Ser	DAA	TAA
Main effects													
PS													
MBM		73.2	77.2[b]	77.5[a]	74.6[a]	69.2[b]	61.4	76.5[b]	65.6[b]	67.9	72.7[b]	69.7[b]	74.0[a]
SBM		79.9	81.6[a]	80.4[a]	78.8[a]	76.6[a]	66.8	83.9[a]	73.9[a]	79.6	79.7[a]	77.0[a]	78.7[a]
CM		64.0	72.9[b]	67.5[b]	67.2[b]	58.8[c]	60.4	78.1[b]	62.2[b]	62.6	61.8[c]	64.4[b]	66.3[b]
Pooled		1.83	1.51	1.68	1.91	1.95	2.44	1.31	2.18	1.92	1.89	1.91	1.78
FF													
	Mash	74.4	79.1[a]	76.8	76.0[a]	71.2[a]	68.1[a]	81.6[a]	71.2[a]	73.4	73.9[a]	73.6[a]	75.4[a]
	Pellet	70.3	75.4[b]	73.4	70.9[b]	65.2[b]	57.6[b]	77.4[b]	63.3[b]	66.7	68.8[b]	67.1[b]	70.5[b]
Pooled		1.49	1.23	1.37	1.56	1.59	1.99	1.07	1.78	1.57	1.55	1.56	1.45
Probabilities, P ≤													
PS		0.00	0.001	0.001	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00
FF		0.06	0.044	0.089	0.03	0.01	0.00	0.00	0.00	0.00	0.02	0.00	0.02
PS × FF		0.68	0.606	0.669	0.66	0.74	0.40	0.58	0.39	0.35	0.79	0.61	0.64

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COMPILATION AND ASSESSMENT OF THE VARIABILITY OF NUTRIENT SPECIFICATIONS FOR COMMONLY USED AUSTRALIAN FEED INGREDIENTS

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Feed represents the primary cost of broiler production, thus the formulation of cost-effective diets that meet broiler nutritional requirements is critical. To ensure this objective is met, nutrient specifications of feed ingredients must be accurately determined. However, Australian broiler nutritionists have expressed concern, as many nutrient specification databases contain dated information or lack Australian specific data. Therefore, the aim of this project was to compile current Australian and global data into a database of nutrient specifications for commonly used feed ingredients within the Australian chicken-meat industry. This database also evaluated the variation within feed ingredients for both Australian and global nutrient specifications, and identified areas which require further study.

Initially, integrated Australian broiler nutritionists were surveyed to identify the most common feed ingredients and key nutrients for consideration in the database. Within this survey, it was identified that information on digestible P, digestible Ca and fibre (all fractions) were rated as important by all nutritionists surveyed (9), as there is presently a lack of data for these specifications. Data were sourced and compiled from a total of 12 companies/databases, and, where data were lacking, references were sought from the literature. Data were collected for 42 ingredients with 102 nutrient specifications per ingredient, where all data were available. The mean value, sample number (n) and standard deviation (SD) were collected for each nutrient specification. From these data, the overall mean, total sample number and average standard deviation reported were calculated for both Australian data and global data. The sample size required to predict the mean value for each nutrient specification to 95% accuracy was also calculated.

Unsurprisingly, within the database there is substantially more global samples than Australian samples, and the number of samples for some Australian ingredients is quite low. Combined with the notable variability observed in the data, it is evident that published Australian sample numbers are not adequate to accurately predict mean values. For example, the mean protein value of wheat for Australian data is 111.6 g/kg (total n = 370; SD = 7.6) and global data is 119.2 g/kg (total n = 37,874; SD = 14.2), representing a 7% difference between the mean protein level. The SD for global data is larger than Australian data, which is expected as there is greater variation in agricultural practices, cultivars, environmental conditions etc., across multiple countries than within one country. However, upon calculation of the sample size required, it is evident that the number of Australian samples (n = 370) is inadequate, as 706 Australian samples are required to determine the mean protein content of wheat to 95% accuracy. When calculated for global data, it is determined that 2,177 samples are required, which is well below the actual sample number within the database (n = 37,874) and thus this figure is reliable. Overall, only 7% of the Australian data compiled meets the sample number required to accurately predict the mean value within 95% accuracy (13% within 90% accuracy), compared to 20% of global data (40% within 90% accuracy). Therefore, greater focus on determining the nutritive value of feed ingredients is required, particularly for Australian data.

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IMPACT OF DIETARY SOLUBLE NON-STARCH POLYSACCHARIDE LEVELS ON THE GASTROINTESTINAL ENVIRONMENT OF YOUNG BROILERS

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The dietary fibre content of poultry diets is frequently neglected during feed formulation, despite the prevalence of fibrous material in feed ingredients and notable impacts of fibre on the gastrointestinal tract environment. The extent of the influence of dietary fibre is dictated by the specific fibre fraction, i.e. primarily its solubility in gastrointestinal environmental conditions, as well as by the age and health status of the bird. Measuring soluble and insoluble non-starch polysaccharides (NSP) enables for more accurate predictions about the response of birds to fibre content compared to conventional crude fibre values (Choct, 2015a). However, there is currently a lack of understanding about NSP values for formulation because inaccurate crude fibre values are still referred to in the formulation of diets (Choct, 2015b). Of particular interest is the impact of soluble NSP (sNSP) due to its impact on digesta viscosity and the microbiota. In this study, broiler chickens were fed either wheat or corn-based diets formulated to contain the same crude fibre and protein content but differing sNSP levels. Four hundred and eighty day-old Ross 308 broilers were randomly allocated into 48 pens in a 2 × 3 factorial arrangement of treatments, each with eight replicates of ten birds. The factors were grain type (corn or wheat) and dietary sNSP level; the sNSP levels for the corn and wheat diets respectively were classified as high (8.5 or 11.5 g/kg), low (5.8 or 9.0 g/kg) or medium (6.3 or 9.7 g/kg) according to the estimated values for corn or wheat-based diet, respectively. Formulated diets contained approximately 2.4 g/kg and 2.5 g/kg crude fibre for corn or wheat-based diets, respectively. On day 14, four birds per pen were randomly selected for measuring the impact of sNSP and cereal grain type on the gizzard, ileum and caeca pH, ileal digesta viscosity and short chain fatty acid (SCFA) concentration in the caeca.

There was a numerical increase in ileal viscosity as sNSP level increased (4.46, 4.04 and 3.95 cP for high, medium and low, respectively, $P = 0.101$). In addition, birds fed the wheat-based diets presented lower ileal pH and 130% increase in ileal viscosity compared to those fed corn-based diets ($P < 0.05$). Furthermore, increasing dietary sNSP level led to the significant reduction in caecal pH (6.04 vs 6.51, $P < 0.05$), suggesting alterations to intestinal microbial fermentation activities. This was evident in groups fed the wheat-based diets, where total SCFA and acetic acid concentration in the caeca were higher in birds fed the diets with low sNSP compared to those fed high sNSP ($P < 0.05$). Interestingly, the concentrations of caecal propionic, isobutyric and valeric acid were consistently greater in the birds fed diets with low sNSP compared to those fed high sNSP (P values < 0.05), with levels in birds fed the medium sNSP diets being not different compared from either group. Additionally, birds fed the corn-based diets exhibited higher valeric and lower succinic caecal concentrations compared to those fed the wheat-based diets (P values < 0.05). This could indicate a significant cereal grain type- and sNSP level-dependent substrate availability for specific microbial fermentation. In conclusion, the findings from this study reiterate that measurements of crude fibre cannot be used to accurately predict utilization of dietary fibre by the birds. It also proves that the sNSP fraction directly impacts the gut environment in young broilers, in terms of intestinal pH, ileal viscosity and fermentation activities of commensal bacteria.

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DEVELOPMENT OF NEAR INFRARED CALIBRATIONS FOR DETERMINATION OF NON-STARCH POLYSACCHARIDE CONTENT IN FEEDSTUFF

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Summary

Reduced use of antibiotics has increased the importance of evaluating the fibre concentration and characteristics in feed ingredients. Better knowledge of fibre composition is needed since the intestinal microbiota largely utilize fibre components as substrates for fermentation. The objective of this paper is to summarize the results of NSP contents of feedstuff samples collected worldwide and develop calibration statistics for prediction of total insoluble and total NSP (sum of soluble and insoluble). Approximately 1,700 feedstuff samples from 24 different countries were collected over 5 years and analysed for NSP composition and solubility. Samples were split into 3 categories which comprised fibrous materials, protein materials and cereals. All samples were ground (1mm particle size) and scanned using a benchtop NIR monochromator spectrometer, covering the spectral range of 400-2500 nm, with a spectral interval of 2 nm, and running Foss Mosaic software. Equations for insoluble NSP showed very good accuracy, with coefficient of determination (R^2) ranging from 86 to 97%, with standard error of cross validation (SECV) being close to standard error of prediction (SEP) values, and RPD (ratio of standard deviation of analysed data by the SEP) between 3 to 6, all of which indicated good models for predicting insoluble NSP. The same scenario was observed for total NSP calibration, with R^2 ranging from 91 to 97% and RPD between 3 to 6. It is possible to use NIR to predict NSP content and fibre characteristics in feed ingredients.

I. INTRODUCTION

Antibiotic growth promoters have been successfully utilized in the past to control gut dysbiosis (Dibner and Richards, 2005). However, poultry production is changing because of consumer and governmental pressure to reduce the use of antibiotics (Cervantes, 2015). Little or no attention is given by nutritionists to understanding the fibre composition in poultry diets and their role in gut health and litter quality. Crude fibre method is largely employed to describe dietary fibre content in poultry nutrition, but this method only captures around 25% of what is considered "true" fibre (Graham et al., 1991; Choct, 2015a). Better knowledge of fibre composition is needed since the intestinal microbiota will largely utilize these components as substrates for fermentation. The fermentation products such as volatile fatty acids can play an important role in gut health and overall metabolism of the host (Józefiak et al., 2004; den Besten et al., 2013; Hervik and Svihus, 2019). As opposed to the term "dietary fibre", the term "analytical dietary fibre" (Kerr and Shurson, 2013; Choct, 2015b) comprises the analysis of the structural carbohydrates by their individual sugars and solubility and gives a much better understanding of the dietary fibre composition of feedstuff. However, the methods used to analyse these fibre components are quite laborious and expensive and, as a result, very few data are available in the literature (Knudsen, 2014; Choct, 2015b; Rostagno et al., 2017). The only table that has reported the non-starch polysaccharides (NSP) contents in feedstuff is the Brazilian Tables for Poultry and Swine (Rostagno et al., 2017). It should be noted that there is a lot of variability in NSP content within the same raw material. Near-infrared reflectance spectroscopy (NIRS) is a well-established method for analysis and quality control of ingredients providing fast and inexpensive analysis of organic compounds (e.g. moisture,

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protein, fat, starch, crude fibre, amino acids, etc), serving as an analytical tool to reduce excess of nutrients while formulating diets (Van Kempen & Simmins, 1997), thereby reducing feed cost and environmental impact of animal production (Ferket et al., 2002). Despite that, very few attempts have been made to create calibrations for NSP determination in feedstuff (Archibald and Kays, 2000; Blakeney and Flinn, 2005; Hollung et al., 2005). The objective of this paper is to summarize the results of NSP contents of feedstuff samples collected worldwide and provide with the calibration statistics for prediction of total insoluble NSP and total NSP (sum of soluble and insoluble NSP).

II. METHODS

Approximately 1,700 feedstuff samples from 24 different countries were collected over 5 years and analysed for NSP at Englyst Carbohydrate Ltd laboratory (Southampton, UK, SO16 7NP). The samples were analysed according to the method proposed by Englyst et al. (1994). Samples were split into 3 categories which comprised fibrous materials (DDGs, wheat bran, rice bran, soy hulls, cassava meal, sunflower meal, etc), protein materials (soybean meal, full fat soy, canola meal, peanut meal, corn gluten meal, etc) and cereals (barley, corn, oat, millet, quinoa, rice, rye and sorghum).

All samples were ground (1mm particle size) and scanned using a benchtop NIR machine (FOSS DS2500) monochromator spectrometer (FOSS A/S Hillerød, Denmark), covering the spectral range 400-2500 nm, with a spectral interval of 2 nm, and running Foss Mosaic software. Six pre-treatments were investigated: raw absorbance spectra, first derivative, and second derivative, each tried without and with Standard Normal Variate (SNV) pre-processing. In the case of two treatments, the SNV was applied after the derivative. The numbers of factors were chosen based on the plot of RMSECV versus the number of factors, observing where the curve starts to flatten out, giving the best RMSEV for the optimum number of factors.

The accuracy of the equations was evaluated through the coefficient of determination (R^2), standard error of cross validation (SECV), standard error of prediction (SEP) and RPD (ratio of standard deviation of analysed data by the SEP).

III. RESULTS

Non-starch polysaccharide results are summarized in Tables 1 and 2. Only ingredients with 10 or more analysis are reported. The coefficient of variation of soluble total NSP ranged from 16 to 101% and that of insoluble total NSP ranged from 6 to 129%.

After removing outliers, the models for fibrous, protein and cereal feedstuff contained 130, 364 and 1053 samples, respectively for NIR calibrations. Overall equations for insoluble NSP showed very good accuracy, with R^2 ranging from 86 to 97%, with SECV being close to SEP values, and RPD between 3 to 6, all of which indicates good models for predicting insoluble NSP. The same scenario was observed for total NSP calibration, with R^2 ranging from 91 to 97% and RPD between 3 to 6.

Table 1 - Non-starch polsaccharide content of cereals (% as fed).

Feedstuff		Xylose	Arabinose	Glucose	Galactose	Mannose	Total
Wheat (n=408)	Insoluble	2.94	1.90	2.34	0.19	0.16	7.53
	Soluble	0.64	0.42	0.29	0.15	0.12	1.62
	Total	3.58	2.32	2.63	0.34	0.28	9.15
Corn (n=345)	Insoluble	2.11	1.51	1.78	0.41	0.08	5.91
	Soluble	0.12	0.12	0.24	0.10	0.06	0.68
	Total	2.23	1.63	2.02	0.51	0.14	6.59
Barley (n=128)	Insoluble	4.55	2.17	3.84	0.27	0.24	11.10
	Soluble	0.34	0.29	3.26	0.13	0.07	4.09
	Total	4.89	2.46	7.10	0.40	0.31	15.19
Sorghum (n=117)	Insoluble	1.07	1.16	2.09	0.21	0.09	4.62
	Soluble	0.06	0.07	0.20	0.07	0.07	0.51
	Total	1.13	1.23	2.29	0.28	0.16	5.13
Rice (n=31)	Insoluble	0.92	1.57	1.80	0.21	0.12	6.49
	Soluble	0.08	0.09	0.12	0.21	0.08	0.58
	Total	1.00	1.66	1.92	0.42	0.20	7.07
Oats (n=25)	Insoluble	10.10	1.98	8.88	0.44	0.17	21.60
	Soluble	0.14	0.16	2.96	0.13	0.05	3.44
	Total	10.24	2.14	11.84	0.57	0.22	25.04
Millet (n=22)	Insoluble	1.65	1.24	1.77	0.29	0.07	5.02
	Soluble	0.08	0.05	0.39	0.08	0.17	0.77
	Total	1.73	1.29	2.16	0.37	0.24	5.79
Triticale (n=18)	Insoluble	4.56	3.67	3.24	0.38	0.34	12.20
	Soluble	0.58	0.44	0.26	0.15	0.08	1.51
	Total	5.14	4.11	3.50	0.53	0.42	13.71

Table 2 - Non-starch polysaccharide content of protein and fibrous ingredients (% as fed).

Feedstuff		Xylose	Arabinose	Glucose	Galactose	Mannose	Total
Soybean Meal (n=181)	Insoluble	1.26	1.81	4.41	2.94	0.54	12.32
	Soluble	0.22	0.63	0.34	1.17	0.45	3.95
	Total	1.48	2.44	4.75	4.11	0.99	16.27
Full Fat Soy (n=96)	Insoluble	1.17	1.64	3.79	2.56	0.45	10.98
	Soluble	0.17	0.52	0.22	0.88	0.33	3.07
	Total	1.34	2.16	4.01	3.44	0.78	14.05
DDGs (n=76)	Insoluble	7.11	5.02	7.51	1.11	1.07	21.93
	Soluble	0.40	0.38	0.44	0.23	0.58	2.19
	Total	7.51	5.40	7.95	1.34	1.65	24.12
Canola Meal (n=30)	Insoluble	1.52	3.35	6.33	1.26	0.37	15.55
	Soluble	0.28	1.09	0.43	0.55	0.28	3.95
	Total	1.80	4.44	6.76	1.81	0.65	19.50
Sunflower Meal (n=23)	Insoluble	5.03	2.50	10.32	0.71	1.13	20.75
	Soluble	0.17	0.80	0.44	0.45	0.34	4.28
	Total	5.20	3.30	10.76	1.16	1.47	25.03
Wheat Bran (n=20)	Insoluble	9.54	6.00	7.22	0.58	0.18	23.62
	Soluble	1.07	0.60	0.61	0.20	0.19	2.83
	Total	10.61	6.60	7.83	0.78	0.37	26.45
Corn Gluten Meal, 60%CP (n=22)	Insoluble	0.44	0.38	0.76	0.22	0.21	2.03
	Soluble	0.33	0.23	0.53	0.14	0.14	1.41
	Total	0.77	0.61	1.29	0.36	0.35	3.44
Corn Gluten Feed, 21%CP (n=18)	Insoluble	3.82	2.74	4.01	0.74	0.35	11.92
	Soluble	0.21	0.18	0.18	0.06	0.12	0.86
	Total	4.03	2.92	4.19	0.80	0.47	12.78
Rice Bran (n=10)	Insoluble	4.26	3.36	6.54	0.91	0.20	15.35
	Soluble	0.13	0.26	0.46	0.22	0.18	1.51
	Total	4.39	3.62	7.00	1.13	0.38	16.86

IV. DISCUSSION

Results of the current study demonstrate the degree of variation in NSP composition between and within ingredients. Fibre has anti-nutritive characteristics but can also improve gut function in monogastrics (De Vries, 2015). Therefore, a better understanding of fibre composition would allow the use of nutritional strategies (e.g. carbohydrase enzymes) to boost fibre fermentation by gut microbiota, thereby influencing the host metabolism positively. However, care should be taken when we refer to fibre hydrolysis and release of simple sugars since this may not be beneficial for the host. As an example, Schutte (1990) showed that, when xylose and arabinose were supplemented to broiler diets, these created performance and litter quality issues. The generation of oligosaccharides is therefore preferable, and can stimulate microbiota fermentative capacity (Broekaert et al., 2011).

The use of NIR to predict NSP content in feed ingredients is possible, as previously demonstrated (Archibald and Kays, 2000; Blakeney and Flinn, 2005; Hollung et al., 2005). Our database covered more ingredients and variation than the previous studies, resulting in robust calibrations that can help nutritionists to better formulate poultry diets.

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AGRIFUTURES AUSTRALIA CHICKEN MEAT – NUTRITION PROGRAM OF RESEARCH

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Summary

This paper provides a brief overview of the four components for this program of research. Three specifically focus on reducing crude protein concentrations in poultry diets, and are complimented by a study on non-starch polysaccharides (NSPs). All have the ultimate aim of identifying opportunities to positively impact economic, environmental, flock health and bird welfare outcomes through changes to wheat and/or sorghum diet formulations.

I. INTRODUCTION

The AgriFutures Australia Chicken Meat Program funds research, development and extension activities for the benefit of the Australian chicken meat industry. Through the process of reviewing the 5-year strategic plan (RIRDC, 2014), the objectives were adjusted to align each objective with a stage of chicken meat production. This included the consolidation of all research related to the live chicken under a single objective, essentially acknowledging that the health, welfare, environmental and nutritional components of raising chickens are intricately linked. To this end, the aim is to invest in projects that are multi-faceted, while encouraging stronger collaborations between researchers and research institutes. Four projects with different nutritional focuses were contracted in 2019 and drawn together under the banner of the 'Nutrition Program of Research'. The projects are separate; however, their milestone schedules, experimental design and outcomes are aligned, and overseen by a single industry steering committee.

II. PROJECT SUMMARIES

a) Optimising amino acid profiles and energy in reduced-protein diets.

In the literature, only two studies by Taherkhani et al. (2005, 2008) and one by Salehifar et al. (2012) have compared 'ideal protein ratios' in meat chickens. While these feeding studies resulted in poor overall bird performance, all three studies report tangible differences in growth performance in response to the ideal protein ratios of diets evaluated. The aim of this project is to investigate the amino acid requirements and validate ideal protein ratios of modern genotype meat chickens, in the context of reduced-protein diets containing high inclusion levels of crystalline amino acids, in experiments in which birds approach or surpass their genetic potential. It focuses on the constraints for least-cost feed formulation including ideal protein ratios, 4th limiting amino acid and energy requirements.

This project will include *in vivo* studies from 1-42 days post-hatch using wheat-based diets with 100 g/kg whole grain for grower diets and 150 g/kg whole grain for finisher diets. The relative importance of each supplemented amino acid will also be ranked. Further, excess fat deposition in meat chickens offered reduced-protein diets is a real challenge. Less energy is required to support intestinal absorption of crystalline amino acids (which are supplemented

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in the diet), compared to energy required for intestinal absorption of amino acids extracted from ingredients in the diet. Consequently, surplus dietary energy is deposited as fat. Therefore, the energy densities of reduced-protein diets could potentially be reduced without compromising performance. This could represent a substantial saving in feed cost.

b) Impact of non-starch polysaccharides in meat chicken diets.

Despite extensive evidence demonstrating the influence of NSPs on a range of processes involved in intestinal health and digestive efficacy (Amerah et al., 2009; Hetland and Svihus, 2001; Jørgensen et al., 1996; Bedford, 1996; Mayne et al., 2007), most commercial nutritionists do not formulate to NSP levels. This is likely because the optimum range in soluble and insoluble NSP level that nutritionists should be aiming for when formulating feed has yet to be defined, from both economic and bird health and welfare viewpoints. This project aims to provide the industry with these values, based on analysis of current commercial diets and research trials.

Furthermore, xylanase (an NSP-ase) supplementation is widely used in poultry diets to break down NSPs in the diet, but there is evidence to suggest that a combination of different NSP-ases may supply further benefits, partially by producing a range of different prebiotic oligosaccharides (Lee et al. 2017; Morgan et al. 2017). Consequently, this project will use both *in vitro* techniques and *in vivo* trials to explore the comparative advantages and disadvantages of NSP-ase cocktails, from both an economic and practical viewpoint, in diets based on wheat or sorghum which have different levels of soluble NSP. Ultimately, the outcome of this project will be improved formulation of diets containing sorghum or wheat.

c) Branched-chain amino acids in wheat-based, crude protein-reduced diets.

Moderate reductions in dietary crude protein (e.g. from 210 g/kg to 175 g/kg) can be achieved without negatively impacting meat chicken growth rate. However, further reductions often have negative implications for feed conversion and increase fat deposition. While numerous factors contribute to this problem, this project aims to specifically investigate the involvement of the branched-chain amino acids (isoleucine, leucine and valine).

Reviews of leucine and branched chain amino acids provide further background to this issue (Li et al. 2011; Zhang et al. 2017). Leucine, in particular, has the potential to increase protein synthesis (Deng et al., 2014) and reduce fat deposition (Duan et al., 2015), and very high levels of leucine in meat chicken diets have produced positive responses (Yamazaki et al. 2006; Chen et al. 2016). Therefore, leucine is suspected to be critical to the implementation of reduced-CP meat chicken diets. A key objective for this project is to determine whether there are benefits to a higher inclusion of leucine in meat chicken diets, and to what extent the benefits rely on increases in leucine being coupled with increases in isoleucine and valine.

Currently, diets based on wheat or sorghum are formulated on the basis of digestible amino acids relative to lysine. However, this project may demonstrate that higher leucine levels are advantageous and that ratios of isoleucine plus valine to leucine will need to be taken into consideration in future.

d) The response of meat chickens to insoluble fibre and exogenous enzymes in reduced-protein diets.

The use of insoluble fibre in poultry diets has been applied in recent years to improve nutrient digestibility and improve intestinal development and bird health (Kheravii et al., 2018a, 2018b, 2018c). The inclusion of insoluble fibre in the diet improves digestion and the function of the intestines by stimulating the gizzard and increasing the retention time of digesta (Svihus, 2011).

Insoluble fibre also improves the secretion of digestive proteases and ultimately results in drier excreta (and therefore fewer wet litter problems). This project aims to determine whether improvements to bird outcomes on reduced-CP diets can be realised through the supplementation of different sources of insoluble fibre and protease in the diet.

This project will include *in vivo* studies from 1-42 days post-hatch to compare wheat and sorghum reduced-CP diets supplemented with added fibre and protease to examine the extent to which insoluble fibre can improve bird performance, and whether addition of protease is necessary, in the context of reduce-CP diets.

IV. DISCUSSION

Outcomes of these projects will be regularly provided through updates via various AgriFutures Australia publications and industry extension activities. There is the potential that the Program will be expanded to include other relevant components as they are raised as priorities by industry and/or identified by researchers.

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DIETARY FAT INCLUSION DECREASES ENDOGENOUS AMINO ACID LOSSES IN BROILER CHICKENS

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Fats and oils are widely used in poultry diets as a source of energy and to increase palatability. Increasing dietary fat level has been reported to improve the apparent ileal digestibility of amino acids (Cowieson and Ravindran, 2008). These improvements may be related, in part, to reduced endogenous amino acid (EAA) losses, but the influence of dietary fat on EAA losses has not been studied in chickens. The present study was carried out to measure the basal EAA losses in male broilers (Ross 308) fed three nitrogen-free diets namely, a control diet with no added fat and test diets with 60 g/kg of either soybean oil or tallow added. Since no protein was fed to the broilers, the assumption is that all nitrogen and amino acids in the ileal digesta will be of endogenous origin and represent the basal losses. Titanium dioxide (5 g/kg) was added to all diets as an indigestible marker. Each diet was assigned to six replicate cages (8 birds per cage) from day 18 to 21. On day 21, the digesta were collected from the lower half of the ileum. The endogenous losses of all amino acids, except cysteine, were higher ($P < 0.05$) in broilers fed diets with no added fat, compared to those fed diets with fat. For all amino acids, there was no significant difference between the endogenous losses in diets supplemented with tallow or soybean oil. Several mechanisms are probably involved in the observed EAA flow responses. First, the addition of fat lowers the passage rate (Mateos et al., 1982), retaining the digesta longer in the intestine, which may increase the digestion and re-absorption of EAA and reduce EAA losses. Endogenous losses represent the net balance between protein ingested plus endogenous proteins and absorbed dietary plus reabsorbed endogenous protein. Thus, it is reasonable to assume that any factor that increases amino acid absorption would favour reductions in EAA flow. Second, reduced EAA losses could be related, at least in part, to morphological changes in the microvilli with fat addition. Goda and Takase (1993) reported that a high-fat diet increased the length of intestinal microvilli, crypt depth and proliferation of microvilli in the intestinal wall. The resulting increase in the surface area could be expected to increase the efficiency of re-absorption of endogenous proteins. Changes in the composition of microbiota with fat addition may be another possible contributing factor. Although the primary area of bacterial activity in the intestine of chickens is the hindgut, the small intestine also contains a significant microbiome presence, particularly associated with mucus layer (Tomas et al., 2016), and many of these species use mucin as their energy and amino acid sources (Pan and Yi, 2014). The above speculations are consistent with the suggestion by Jenkins and Thompson (1992) that higher dietary fat content might increase amino acid absorption in the proximal intestinal segments. The present data indicate that the inclusion of fat in broiler diets decreases endogenous amino acid losses and implies that dietary fats have beneficial effects beyond energy contribution and palatability.

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ENDOGENOUS AMINO ACID FLOWS ARE INFLUENCED BY AGE OF BROILER CHICKENS

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During the process of digestion and absorption of ingested feed in poultry, significant losses of endogenous amino acids (EAA) occur from various digestive secretions, mucoproteins and the epithelial cells lining the gastrointestinal tract (GIT). Several factors such as the development of GIT and changes in dry matter intake (DMI) with advancing age, type of bird, method of euthanasia and ileal digesta collection may influence EAA loss. Correction for these inevitable losses is necessary to standardise amino acid (AA) digestibility values. The present study was carried out to measure the basal EAA loss in male broilers (Ross 308) at different ages (d 7, 14, 21, 28, 35 and 42), using a nitrogen-free diet (NFD). The assumption is that, since no protein is fed, all nitrogen and AA in the ileal digesta are of endogenous origin and represent the basal losses. The NFD was composed of maize starch (842 g/kg), fibre source (cellulose; 50 g/kg), soybean oil (50 g/kg) and mineral and vitamin premix (53 g/kg). Titanium dioxide (5 g/kg) was also added to the NFD as an indigestible marker. The NFD diet was fed to six replicate cages housing 14 (d 7), 12 (d 14), 10 (d 21), 8 (d 28), 8 (d 35), and 6 (d 42) birds per cage for four days prior to digesta collection. Following euthanisation by intravenous injection of sodium pentobarbitone, the digesta was collected by gentle flushing with distilled water from the lower half of the ileum. The basal EAA flow was calculated as grams per kilogram of DMI (g/kg DMI). Data were analysed by using general linear models procedure of SAS (SAS Institute, Inc., Cary, NC) with cage means as the experimental unit. Orthogonal polynomial contrasts (linear and quadratic) were used to compare the treatment means. The flow of ileal endogenous N and all AA, on a DMI basis, decreased quadratically ($P < 0.05$ to 0.001) as birds grew older. The values of ileal endogenous loss of N and total endogenous loss of all AA (TAA), when expressed per kg of DMI, were higher ($P < 0.01$) at d 7 compared to other ages. The values for ileal endogenous N and total EAA losses at d 7 were 3.60 and 12.9 g/kg DMI, respectively. In agreement with the present findings, Adedokun et al. (2007) recorded approximately two times higher total EAA loss in broilers at d 5 compared to d 15 and 21. However, the endogenous flow values for N and TAA were similar at d 14, 21, 28 and 35. The lowest endogenous loss for TAA (4.48 g/kg DMI) was determined at 42 d. When an NFD is fed, the source of EAA is largely mucoproteins (Lemme et al., 2004). Decreased mucin secretion, increased endogenous protein digestion and absorption, and increased DMI with age may account for the lower EAA secretion in older birds (Ravindran and Bryden, 1999). Current data suggest that specific age-related values for EAA loss should be used to standardise AA digestibility coefficients in broilers.

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THE COST OF DEAMINATION IN REDUCED-CRUDE PROTEIN BROILER DIETS

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Summary

The proposal is that the 'cost of deamination' may be an important contributing factor to compromised growth performance of broiler chickens offered reduced-CP diets, which stems from deamination of excess, imbalanced amino acids in reduced-CP diets. Deamination releases ammonia, which is detoxified in a condensation reaction with glutamic acid to yield glutamine, which is then excreted via the Krebs cycle as uric acid. However, excessive plasma ammonia levels may accumulate when ammonia is not adequately detoxified to compromise growth performance.

I. INTRODUCTION

Reductions in crude protein (CP) contents of broiler diets have been realised for decades by routine additions of unbound (synthetic or crystalline) methionine, lysine and threonine, and are likely to continue as inclusion costs for the balance of amino acids become more feasible. Reduced-CP diets have the potential to provide environmental advantages from attenuated nitrogen and ammonia outputs (Nahm, 2007), bird welfare, from enhanced litter quality and lower incidences of foot-pad dermatitis (Dunlop et al., 2016), and flock health, from less undigested protein entering the large intestine to fuel the proliferation of potential pathogens (Wilkie et al., 2005). However, there appears to be a threshold where tangible CP reductions of more than 3 to 4 percentage units negatively influence growth performance, especially FCR, and this is associated with increased fat deposition (Belloir et al., 2017). Many possible explanations have been advanced and numerous strategies evaluated in attempts to lower this threshold, but the problem of compromised growth performance, especially in wheat-based diets, remains. However, the 'cost of deamination' may be contributing towards compromised growth performance in birds offered reduced-CP diets.

II. BACKGROUND

Dietary amino acid imbalances generated inferior growth performance in the Snetsinger and Scott (1961) study. This negative impact was partially alleviated by glycine, which was attributed to glycine enhancing the excretion of excess nitrogen (N) via the uric acid cycle. However, the effects of glycine and glutamic acid were shown to be additive in this context (Maddy et al., 1960). Additions of imbalanced amino acid mixtures to low protein diets was investigated by Hill and Olsen (1963) who concluded that the resultant depressions in weight gain stemmed from deamination of relatively large quantities of amino acids. The blend of unbound and protein-bound amino acids in reduced-CP diets almost certainly leads to amino acid imbalances at sites of protein synthesis, and any surplus of amino acids require deamination. The principal mechanism for this is oxidative deamination in the liver, which generates ammonia that demands detoxification. Ammonia detoxification is a condensation reaction in which ammonia and glutamic acid are converted to glutamine. The reaction is driven by glutamine synthetase, which is present in both mammalian (Hakvoort et al., 2017) and avian (Watford and Wu, 2005) species. In poultry, glutamine is incorporated into the Krebs uric acid cycle and N is excreted as uric acid, which is an energy consuming process that requires glycine inputs (Salway, 2018), where serine, and possibly threonine, may serve as glycine precursors.

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Interestingly, Mapes and Krebs (1978) investigated the rate-limiting factors in hepatic uric acid synthesis in chickens and suggested that glutamate and glutamine are fundamental to ammonia detoxification and N excretion. Energy considerations are also involved in these pathways as synthesis and excretion of one molecule of uric acid involves the loss of one glycine molecule, which has the potential to yield 12.5 molecules of ATP (Salway, 2018).

Free amino acid concentrations in portal and systemic plasma in birds offered 21.5% and 16.5% CP wheat-based diets were determined by Yin et al. (2019). In this instructive study, portal concentrations were higher than systemic concentrations, but both followed similar patterns. The reduction in dietary CP generated an average 30.9% increase in glutamine levels, which may have resulted from increased condensations of ammonia plus glutamic acid into glutamine. Concentrations of glycine equivalents declined by 23.6% which may reflect increased inputs of glycine and serine into the Krebs uric acid cycle. In contrast, threonine concentrations rose by 28.0%, which is not indicative of threonine being a glycine precursor as glycine levels decreased by 27.7% in this study.

Ammonia intoxication arises when an excess of ammonia is produced or its removal is retarded and ammonia interferes with metabolism. Indeed, high blood ammonia levels depressed feed intakes via mechanisms involving the central nervous system in rats offered amino acid imbalanced diets (Noda, 1975; Noda and Chikamori, 1976). Thus, there is the possibility that amino acid imbalances and inadequacies in reduced-CP diets result in deamination of excess amino acids and, in turn, lead to the accumulation of ammonia in birds, which has negative impacts. Reduced-CP diets contain substantially less glutamic acid, glutamine and glycine than conventional diets so one implication is that inadequate glutamic acid concentrations in reduced-CP diets are impeding ammonia detoxification and inadequate concentrations of glycine, or glycine equivalents, are retarding the Krebs uric acid cycle and uric acid excretion. Alternatively, imbalances of unbound and protein-bound amino acids in reduced-CP diets may be generating substantial excesses of amino acids that require deamination that is not being adequately met.

III. SUPPORTIVE DATA

Data generated by both Namroud et al. (2008) and Ospina-Rojas et al. (2014) provide support for the proposal that the hepatic oxidative deamination of amino acids with the liberation of ammonia in reduced-CP broiler diets may result in excessive plasma ammonia concentrations, which has negative impacts on growth performance. A significant increase in systemic plasma ammonia concentrations of 14.5% (0.71 versus 0.62 mg/100mL) following a reduction in dietary CP from 230 to 170 g/kg was reported in broiler chickens by Namroud et al. (2008). Moreover, it may be deduced from this study that there were negative linear regressions between mean systemic plasma ammonia concentrations and 28-day body weights ($r = -0.982$; $P < 0.001$) and 10 to 28-day feed intakes ($r = -0.962$; $P < 0.001$) in broiler chickens offered eight dietary treatments with different CP levels and inclusions of unbound amino acids. In addition, there was a quadratic relationship ($r = 0.941$; $P = 0.004$) between ammonia concentrations and FCR as illustrated in Figure 1.

Subsequently, Ospina-Rojas et al. (2014) recorded systemic plasma concentrations of ammonia and the transition from 220 to 190 g/kg CP diets significantly increased ammonia concentrations by 59.4% (7.27 versus 4.56 mg/dl). Ten different combinations of valine, isoleucine, arginine and glycine were added to the 190 g/kg CP diet to provide a total of twelve dietary treatments. It may be deduced that there was a quadratic relationship ($r = 0.799$; $P = 0.015$) between mean ammonia plasma concentrations and 21-day weight gain, where increasing ammonia concentrations were associated with a decline in weight gains, as shown in Figure 2.

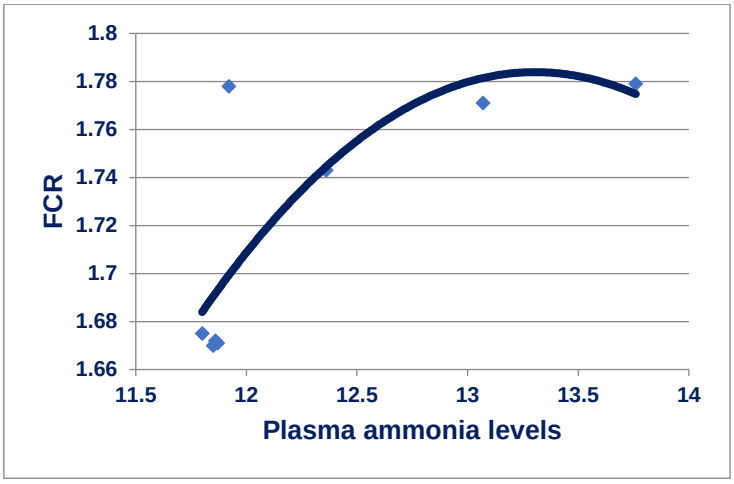

Figure 1 - Quadratic relationship (r = 0.941; P = 0.004) between systemic NH_3 levels (mg/100 mL) and 10 to 28-day FCR in broiler chickens (Namroud et al. 2008).

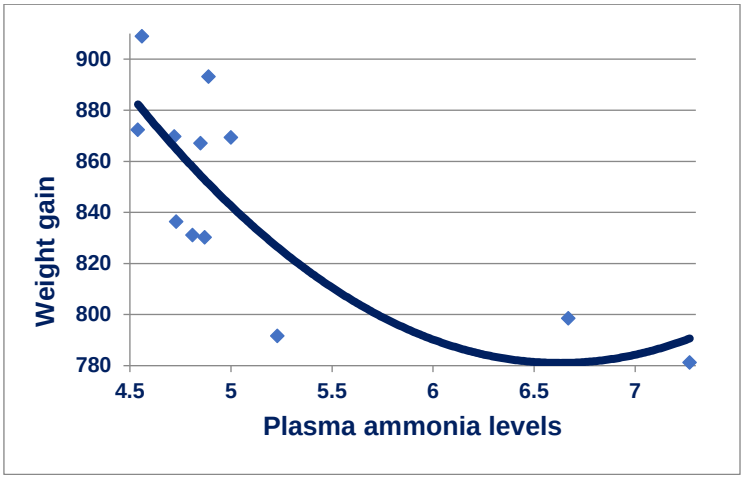

Figure 2 - Quadratic relationship (r = 0.799; P = 0.015) between systemic NH_3 levels (mg/dl) and 21-day weight gains in chickens (Ospina-Rojas et al. 2014).

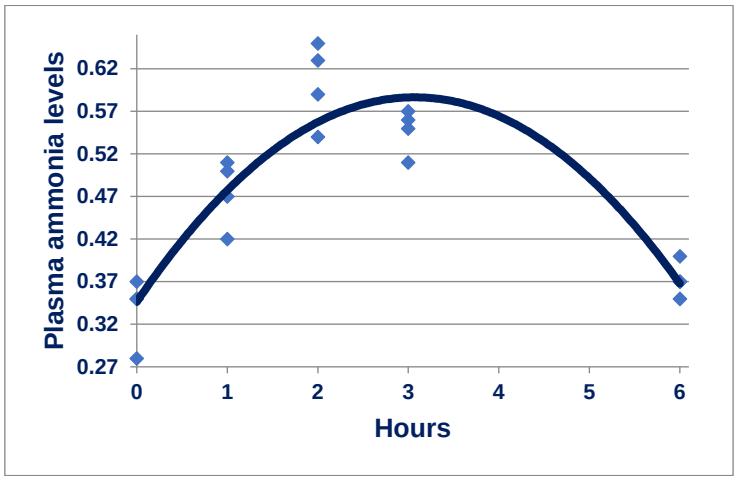

Figure 3 - Quadratic relationship (r = 0.919; P < 0.001) between sampling time and systemic NH_3 levels (mg/100 ml) where y = 0.346 + 0.157*hours – 0.025*$hours^2$ (Okumura and Tasaki, 1969).

IV. DETERMINATION OF PLASMA AMMONIA CONCENTRATIONS

The determination of ammonia concentrations in portal or systemic plasma *per se* is straightforward but is complicated by the fact that plasma ammonia concentrations are volatile over the sampling time duration. Okumura and Tasaki (1969) documented the volatility of systemic portal ammonia concentrations in poultry and it may be deduced that these researchers detected a quadratic relationship ($r = 0.919$; $P < 0.001$) between elapsed sampling time and systemic ammonia plasma concentrations in birds offered diets containing 150, 200 300 and 400 g/kg casein. It may be calculated from the regression equation that ammonia plasma levels were 0.35 mg/100 ml at zero hours, peaked at 0.59 mg/100 ml after 3.14 hours and had returned to 0.39 mg/100 after six hours, as shown in Figure 3. This means that the interpretation of plasma ammonia concentrations will be best addressed by an ANCOVA, or an analysis of covariance, which is a blend of standard analyses of variance with quadratic regressions. The accurate interpretation of plasma ammonia concentrations may prove pivotal in investigations into the likely cost of deamination and, in extreme cases, the possibility of ammonia toxicity in birds offered reduced-CP diets.

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BRANCHED-CHAIN AMINO ACIDS: POTENTIAL ANTAGONISMS IN PRACTICAL FORMULATION

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Summary

The branched-chain amino acids (BCAA) are essential amino acids that become limiting after Lys, Met, and Thr in broiler diets. Specifically, Val or Ile are potentially the 4th limiting amino acid in practical diets. Therefore, determination of the requirements for the BCAA are necessary considering the increased interest in reduced protein diets. A series of studies were undertaken at the Arkansas Agricultural Experiment Station to assess the potential interactions among BCAA in broilers. It was determined that Val is likely the 4th limiting amino acid and that Leu levels may be at potentially excessive levels in practical diets. Due to these potentially excessive levels of Leu, competition between Ile and Val can lead to significant increases in feed conversion ratio and reductions in breast meat yield. Antagonism between Ile and Val was also observed in all four studies except one in which Leu to Lys ratio was reduced to 110. Future research assessing BCAA interactions in different broiler ages and strains is warranted.

I. INTRODUCTION

Interactions among the branched-chain amino acids (BCAA) have long been observed in poultry (D'Mello and Lewis, 1970; Tuttle and Balloun, 1976; Mendonca and Jensen, 1989), but their effects on broiler performance have been contested with various researchers reporting no effects of BCAA interactions on broiler performance in practical diets (Burnham et al., 1992; Barbour and Latshaw, 1992; Waldroup et al., 2002). Early studies concerning the BCAA have attributed the adverse effects of BCAA interactions to excess Leu which decreases appetite resulting in decreased bodyweight (BW) gain (D'Mello and Lewis, 1970). Expanding on these findings, Allen and Baker (1972) found that when Leu was in excess, the efficacy of dietary Ile and Val decreased and could exacerbate reductions in performance due to either Ile or Val being 4th limiting. This hypothesis was supported by the findings of Jackson and Potter (1984), who found that in the event of a Leu excess, additions of Ile could further reduce performance when Val was 4th limiting due to a reciprocal antagonism. This antagonism was again identified in 1989 when Mendonca and Jensen observed reductions in performance when Ile was added to a diet with marginal Val but, when Ile and Val were added in concert, no adverse effects were observed. Due to the location of Ile and Val as the 4th limiting amino acids in maize-soybean meal diets with or without an animal protein meal, respectively (Kidd and Hackenhaar, 2005), it is of importance to not only understand the antagonism between these two amino acids but also their connection with Leu as crude protein (CP) is reduced in commercial diets and these amino acids are supplemented as feed grade amino acids to allow for crude protein reduction.

II. RECENT RESEARCH

Recent research at the Arkansas Agricultural Experiment Station has attempted to characterize interactions among the BCAA and evaluate Val impact on live performance and carcass traits. Four studies were conducted to determine: the 4th limiting amino acid in maize-soybean meal

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diets, Val requirements for live performance and carcass traits, and assess potential interactions or antagonisms among the BCAA. These studies served to provide new data points concerning BCAA manipulation at levels currently used in the poultry industry.

Cobb 500 male broilers were used in an amino acid deletion assay to determine the 4th limiting amino acid for broilers during a 15 to 35 d grower period. A positive control diet was formulated to 186.5 g/kg of CP but was fortified with crystalline amino acids in such a way that its amino acid profile was similar to that of a 202.9 g/kg CP diet. A negative control diet was created by removing all supplemental amino acids. Eight subsequent deletion diets (Val, Ile, Leu, Trp, Arg, His, Phe, and Gly+Pro) were then made by refortifying the negative control with all crystalline amino acids except the one being tested. Reductions (P = 0.05) in BW gain were observed for birds fed diets devoid in Val compared with birds fed the positive control. Feed conversion ratio was decreased (P = 0.01) for birds fed diets devoid of Ile compared with birds fed the positive control. No differences were observed for any carcass traits as a result of deletion of any amino acid.

Cobb 500 male broilers were also used to access BCAA interactions using Box-Behnken Design (BBD) in two experiments. For both experiments, Val and Ile ratios to Lys were maintained at 65, 75, and 85 and 58, 66, and 74, respectively. The first BBD experiment included Leu as the third factor and it was included at ratios to Lys of 110, 130, and 150 for a 15 to 34 d grower period. The second BBD study included Gly as the third factor and it was included at ratios of total Gly+Ser to Lys of 131, 151, and 171. Both experiments evaluated live performance and carcass traits. In Experiment 1, interactions between Ile and Val were found for feed conversion ratio (P < 0.01, Figure 1) and breast meat yield (P < 0.05, Figure 2). Interactions were also observed between Leu and Val for breast meat yield (P = 0.03). In Experiment 2, no interactions were observed between Ile and Val, but a significant interaction between Gly+Ser and Val was observed for leg quarter yield (P < 0.05).

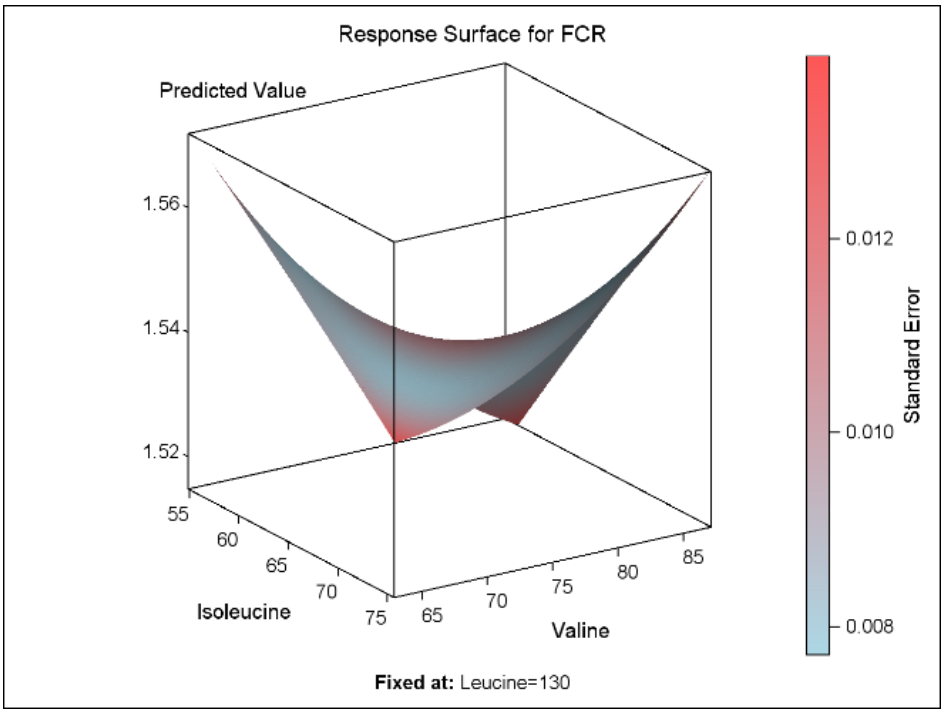

Figure 1 - Feed conversion ratio (FCR, g:g) of Cobb MV × 500 male broilers fed gradient levels of Isoleucine and Valine. Dietary Leucine held constant at Leucine/Lysine ratio of 130. P<0.01.

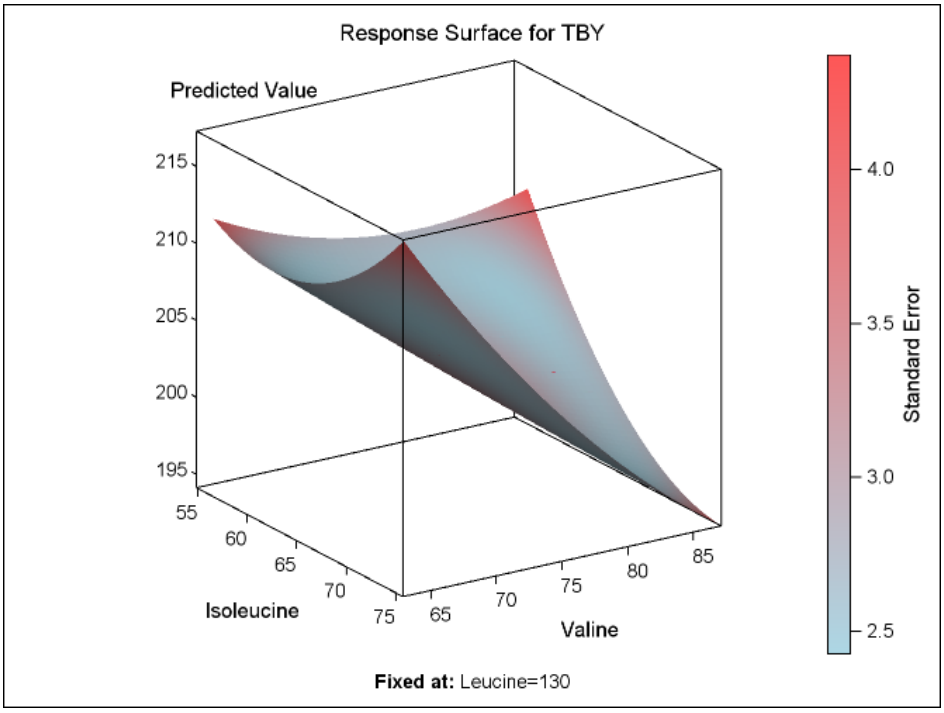

Figure 2 - Breast meat yield (TBY, g/kg) of Cobb MV × 500 male broilers fed gradient levels of Isoleucine and Valine. Dietary Leucine held constant at Leucine/Lysine ratio of 130. P<0.05.

In the final study, Cobb 500 male and female broilers were used to determine the Val requirement during a 15 to 35 d grower period. A Val deficient diet was formulated to titrate Val to Lys ratios of 52, 64, 71, 78, 85, and 92. Additionally, Ile was increased from 66 to 70 in an additional 78 Val diet to observe any potential interactions between Ile and Val. Cubic responses were observed for feed conversion ratio in both male (P = 0.01) and female broilers (P = 0.02), and a linear response (P = 0.05) of increasing body weight gain to increasing dietary Val was observed in females. Valine requirement for feed conversion ratio was estimated for males and females using 95% of the quadratic response and was determined to be Val to Lys ratios of 73 and 72, respectively. No linear, quadratic, or cubic response was observed for any processing yield in male or female broilers. Breast meat yield was reduced for male broilers fed diets with increased Ile compared to those fed the lower level of Ile. No other response was observed as a result of increased Ile.

III. DISCUSSION

The aforementioned responses align with those currently seen in the literature as a response to BCAA interactions, although to a lessened degree. The identification of Val as the 4th limiting amino acid agrees with previous literature (Fernandez et al., 1994; Kidd and Hackenhaar, 2005) and the observation of the reduction of Ile improving feed conversion agrees with the findings of Mendonca and Jensen (1981) that there exists a potential antagonism between Ile and Val when either is the 4th limiting amino acid.

Combined results of the BBD studies give insight into the interconnected nature of the BCAA. In both studies, no linear or quadratic effects were observed for any measured live performance or processing measurement; but when considering potential interactions, effects were observed for both live performance and processing measurements. Also, the fact that the interactions between Val and Ile were not observed in both experiments suggests that Leu levels may have been in excess and, therefore, caused interactions to occur between Ile and Val. Classically, Leu has been shown to have detrimental effects when included at levels far

beyond the bird's requirement (D'Mello and Lewis, 1970) but when high levels of Leu were used in practical diets, concerns about the detrimental effects of high Leu seem unfounded (Waldroup et al., 2002). Indeed no direct effects of Leu were observed in the first BBD study but the competitive nature of Ile and Val observed in the first study were not duplicated in the second, aligning with the theory of Allen and Baker (1974) that excess Leu impairs the efficacy of Val and Ile.

In the final study, only the feed conversion was affected enough to allow for the estimation of a requirement which agrees with previous Val titrations for this growout period (Campos et al., 2009; Morais et al., 2010; Naseimento et al., 2016). The estimated requirements reported herein for males and females are reflective of values established by Farran and Thomas (1990). The reduction in breast meat yield observed in male broilers as a response to increasing dietary Ile was similar to that observed in the previous BBD studies, but similar responses have not been reported in the literature (Dozier et al., 2012).

In conclusion, there do appear to be interactions among the BCAA in practical diets fed to Cobb MV × 500 broilers, but the extent of their effect may be more subtle than the drastic changes in body weight seen in the literature. The Val titration study found no effects on processing yields, which agrees with previous Val titration studies, whereas they were observed in the BBD study and a similar response surface study by Ospina-Rojas et al. (2017). Therefore, the lack of significant linear or quadratic effects in the BBD studies, as well as in the Val titration, highlights the importance of evaluating the BCAA as a whole, instead of as individual amino acids.

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EXOGENOUS PHYTASE ENHANCES WEIGHT GAINS AND INCREASES ILEAL AMINO ACID DISAPPEARANCE RATES IN BROILER CHICKENS

P.V. CHRYSTAL[1,2], P.H. SELLE[1], S.Y. LIU[1], J.Y. LI[3] and Y.M. BAO[4]

Summary

The present study determined phytase efficacy in broiler chickens offered wheat-based diets from 1 to 21 days post-hatch. Exogenous phytase significantly increased weight gains and feed intakes and increased apparent ileal disappearance rates of the sixteen amino acids assessed by a total of 4.67% (10.31 versus 9.85 g/bird/day; $P < 0.001$).

I. INTRODUCTION

The inclusion of exogenous phytases in diets for broiler chickens has developed into a routine practice because of their positive phosphoric and extra-phosphoric effects. On one hand phytase liberates phytate-bound phosphorus (PP) which is found in all plant-sourced feedstuffs as the dominant P fraction. On the other, phytase enhances amino acid (AA) digestibilities, essentially because they prevent the *de novo* formation of binary protein-phytate complexes in the gut (Selle et al., 2012). Whilst several phytases are available in Australia, the exogenous phytase used in this study is a new entrant into the Australian market. Thus, the purpose of this study was to evaluate this exogenous phytase in respect of growth performance, bone mineralisation and AA disappearance rates from the ileum in broiler chickens offered broiler starter crumbles from 1 to 21 days post-hatch using typical Australian-sourced feed ingredients.

II. METHODOLOGY

The experimental protocol was approved by the Animal Ethics Committee of The University of Sydney. A total of 960 day-old male Ross 308 birds were randomly assigned to 4 treatments with 6 replicates of 40 birds per pen. The design was a 2 × 2 factorial array of treatments with wheat-based diets, either a positive (PC) or a negative control (NC), without and with a bacterial (*Escherichia coli*) phytase expressed in *Pichia pastoris* (Microtech 10000 plus; Guandong VTR Biotech Co Ltd) at an inclusion of 1000 FTU/kg. The NC diets were formulated with reductions of 1.6 g/kg Ca, 1.3 g/kg P and 0.3 g/kg Na relative to the PC diets as shown in Table 1. The diets were steam-pelleted at a conditioning temperature of 80ºC and phytase activities analysed. The two non-supplemented diets contained an average of 354 FTU/kg whilst the two supplemented diets contained 1134 FTU/kg phytase activity, post steam-pelleting. Growth performance was monitored from 1 to 21 days post-hatch. At the conclusion of the starter phase, six birds per replicate pen were euthanased and digesta samples from the distal ileum and toes taken to determine apparent AA digestibility coefficients (ADC) and bone mineralisation by standard procedures. AA disappearance rates (g/bird/day) were calculated from the following equation:

$$\text{disappearance rate}_{(g/bird/day)} = \text{dietary AA concentration}_{(g/kg)} \times \text{daily feed intake}_{(g/bird)} \times \text{ADC}$$

Experimental data was subjected to two-way analyses of variance using the IBM® SPSS® Version 24 statistical package.

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Table 1 - Composition and nutrient specifications of starter diets (1 to 21 days post-hatch).

Ingredient (g/kg)	Positive control	Positive control plus phytase	Negative control	Neg. control plus phytase
Wheat	507	507	525	525
Soybean meal	294	294	290	290
Canola meal	75	75	75	75
Soy oil	66	66	60	60
L-lysine HCl	2.71	2.71	2.76	2.76
D,L-methionine	2.66	2.66	2.63	2.63
L-threonine	1.24	1.24	1.24	1.24
L-valine	0.40	0.40	0.38	0.38
Sodium chloride	2.23	2.23	2.19	2.19
Sodium bicarbonate	2.61	2.61	1.57	1.57
Limestone	6.13	6.13	7.27	7.27
Dicalcium phosphate	17.62	17.62	8.10	8.10
Xylanase	0.05	0.05	0.05	0.05
Phytase	-	0.10	-	0.10
Choline chloride (60%)	0.90	0.90	0.90	0.90
Celite	20.0	20.0	20.0	20.0
Sand	0.10	-	0.10	-
Vitamin-mineral premix[1]	2.00	2.00	2.00	2.00
Specifications				
Metabolisable energy	12.77	12.77	12.77	12.77
(MJ/kg)	232	232	232	232
Crude protein	7.60	9.20	6.00	7.60
Calcium	6.50	6.50	4.94	4.94
Total phosphorus	3.80	5.10	2.50	3.80
Available phosphorus	2.20	2.20	2.21	2.21
Phytate phosphorus	4.30	4.30	2.73	2.73
Non-phytate phosphorus	1.80	2.10	1.50	1.80
Sodium	9.38	9.38	9.38	9.38
Potassium	2.50	2.50	2.50	2.50
Chloride	248	261	234	247
DEB (mEq/kg)				
Digestible amino acids	12.12	12.12	12.12	12.12
Lysine	5.61	5.61	5.61	5.61
Methionine	8.00	8.00	8.00	8.00
Threonine	2.61	2.61	2.61	2.61
Tryptophan	8.24	8.24	8.24	8.24
Isoleucine	14.38	14.38	14.38	14.38
Leucine	13.08	13.08	13.08	13.08
Arginine	9.57	9.57	9.57	9.57
Valine				

[1]The vitamin-mineral premix supplied per tonne of feed: [MIU] retinol 12, cholecalciferol 5, [g] tocopherol 50, menadione 3, thiamine 3, riboflavin 9, pyridoxine 5, cobalamin 0.025, niacin 50, pantothenate 18, folate 2, biotin 0.2, copper 20, iron 40, manganese 110, cobalt 0.25, iodine 1, molybdenum 2, zinc 90, selenium 0.3.

III. RESULTS AND DISCUSSION

Overall growth performance of birds in the present study compared favourably with the 2019 Ross 308 performance objectives for 1 to 21 days post-hatch. The transition from PC to NC diets depressed weight gain by 2.11% (976 versus 997 g/bird; $P < 0.05$) and phytase increased weight gain by 1.33% (988 versus 975 g/bird; $P < 0.025$) as shown in Table 2. Phytase similarly increased weight gains in both PC and NC diets by 2.44% and 2.18%, respectively and increased feed intake by 1.82% (1175 versus 1154 g/bird; $P < 0.05$). The transition from PC to

NC diets compromised FCR by 1.71% (1.192 versus 1.172; P < 0.005). The 1.46% mortality rate was not influenced by treatment. A treatment interaction (P < 0.05) was observed for toe ash but the likelihood is that the overall result of 12.84% was indicative of adequate bone mineralisation. Dietary treatments on apparent ileal disappearance rates of sixteen AA are shown in Table 3 where treatment interactions were observed for lysine (P < 0.05) and proline (P < 0.05). Given this caveat, phytase significantly increased all AA disappearance rates culminating in a 4.67% increase (10.31 versus 9.85 g/bird/day; P < 0.001) in total disappearance rates. The transition from PC to NC diets significantly increased disappearance rates of arginine, histidine, leucine, methionine and threonine but decreased that of tyrosine.

Table 2 - Effects of dietary treatments on growth performance from 1 to 21 days post-hatch and bone mineralisation expressed as toe ash.

Treatment		Weight gain	Feed intake	FCR	Mortalities	Toe ash
Diet	Phytase	(g/bird)	(g/bird)	(g/g)	(%)	(%)
Positive	0 FTU/kg	985	1158	1.176	2.08	12.90[b]
control	1000 FTU/kg	1009	1177	1.168	2.08	13.35[b]
Negative	0 FTU/kg	965	1152	1.194	0.83	12.72[a]
control	1000 FTU/kg	986	1173	1.189	0.83	12.39[a]
SEM		9.298	9.032	0.0060	0.802	0.1678
PC		997[b]	1168	1.172[a]	2.08	13.12
NC		976[a]	1162	1.192[b]	0.83	12.55
0 FTU/kg		975[a]	1154[a]	1.185	1.46	12.81
1000 FTU/kg		998[b]	1175[b]	1.179	1.46	12.87
Significance (P =)						
Diet		0.034	0.543	0.004	0.135	0.003
Phytase		0.023	0.035	0.296	1.000	0.713
Diets x Phytase interaction		0.887	0.949	0.103	1.000	0.030

[ab] Means within columns not sharing a common superscript are significantly different at the 5% level of probability

Phytase increased ileal AA disappearance rates or, effectively, their uptakes from the small intestine. The likelihood this is the result of phytase enhancing both protein digestion by retarding the formation of binary protein-phytate complexes which are refractory to pepsin digestion and AA absorption. Phytase has been shown to have positive impacts on sodium pump activity along the small intestine in poultry, which would facilitate the co-absorption of AA and sodium via several Na^+-dependent transport systems (Selle et al., 2012; Akter et al., 2019).

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Table 3 - Effects of dietary treatments on apparent disappearance rates (g/bird/day) of amino acids at 21 days post-hatch.

Treatment		Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenyl-alanine	Threonine	Valine
Diet	Phytase									
PC	0 FTU/kg	0.648	0.274	0.446	0.764	0.610[a]	0.219[b]	0.513	0.388	0.495
	1000 FTU/kg	0.689	0.289	0.476	0.804	0.641[b]	0.211[a]	0.538	0.415	0.507
NC	0 FTU/kg	0.669	0.283	0.457	0.789	0.629[b]	0.213[ab]	0.525	0.402	0.505
	1000 FTU/kg	0.705	0.296	0.475	0.816	0.695[c]	0.232[c]	0.544	0.432	0.531
SEM		0.0065	0.0027	0.0048	0.0082	0.0062	0.0025	0.0054	0.0047	0.0056
Positive control		0.668[a]	0.282[a]	0.461	0.784[a]	0.625	0.215	0.526	0.402[a]	0.507
Negative control		0.687[b]	0.289[b]	0.466	0.802[b]	0.662	0.222	0.535	0.417[b]	0.518
0 FTU/kg		0.658[a]	0.278[a]	0.452[a]	0.777[a]	0.619	0.216	0.519[a]	0.396[a]	0.500[a]
1000 FTU/kg		0.697[b]	0.292[b]	0.475[b]	0.810[b]	0.668	0.222	0.541[b]	0.423[b]	0.525[b]
Significance (P =)										
Diet (D)		0.011	0.010	0.356	0.037	< 0.001	0.010	0.103	0.004	0.057
Phytase (P)		< 0.001	< 0.001	< 0.001	0.001	< 0.001	0.031	0.001	< 0.001	< 0.001
DxP interaction		0.752	0.690	0.199	0.427	0.012	< 0.001	0.604	0.780	0.814

Treatment		Alanine	Aspartic acid	Glutamic Acid	Glycine	Proline	Serine	Tyrosine	Total
Diet	Phytase								
PC	0 FTU/kg	0.400	0.888	2.357	0.409	0.593[a]	0.486	0.247	9.74
	1000 FTU/kg	0.420	0.915	2.434	0.420	0.625[b]	0.512	0.267	10.21
NC	0 FTU/kg	0.403	0.915	2.393	0.417	0.614[b]	0.498	0.244	9.96
	1000 FTU/kg	0.424	0.955	2.427	0.437	0.676[c]	0.513	0.258	10.41
SEM		0.0047	0.0104	0.0209	0.0051	0.0063	0.0056	0.0027	0.0999
Positive control		0.410	0.915	2.395	0.420	0.609	0.499	0.257[b]	9.98[a]
Negative control		0.414	0.935	2.410	0.427	0.645	0.505	0.251[a]	10.18[b]
0 FTU/kg		0.402[a]	0.901[a]	2.375[a]	0.413[a]	0.604	0.492[a]	0.246[a]	9.85[a]
1000 FTU/kg		0.422[b]	0.949[b]	2.430[b]	0.434[b]	0.650	0.513[b]	0.263[b]	10.31[b]
Significance (P =)									
Diet (D)		0.454	0.074	0.496	0.188	< 0.001	0.294	0.033	0.050
Phytase (P)		< 0.001	< 0.001	0.016	0.001	< 0.001	0.001	< 0.001	< 0.001
DxP interaction		0.986	0.538	0.317	0.873	0.025	0.921	0.304	0.930

[a,b & c] Means within columns not sharing a common superscript are significantly different at the 5% level of probability.

IMPROVING UTILISATION OF SOYA BEAN AND CANOLA MEALS WITH THE USE OF MULTIENZYME SOLUTION IN BROILER CHICKEN DIETS

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Soybean meal (SBM) and canola meal (CM) are the main contributors to the protein fraction in poultry diets worldwide. However, these quality vegetable protein meals contain low molecular weight sugars (raffinose and stachyose) and cell wall pectic non-starch polysaccharides (NSPs), cellulose and hemicellulose (e.g. xyloglucans and mannans) which can be problematic. These NSPs are notorious for their water-holding capacity, increasing viscosity in the small intestine, impairing nutrient digestion and absorption and consequently inducing wet litter problems. When NSP-multienzyme combinations targeting specific fiber substrates are applied, the pectic and hemicellulose fiber components of SBM and CM walls are degraded, releasing valuable entrapped nutrients (Pedersen et al., 2015, 2017). The present study was conducted to ascertain if application of NSP-multienzyme blends, designed for specific fiber substrates, improve performance in broilers offered SBM and CM wheat-based diets. The positive control (PC) treatments were either SBM alone (PC1) or mixed with CM in the diets at 10, 18 and 27.5%, for starter, grower and finisher periods respectively, (PC2). In order to generate the negative control treatments for birds to underperform, these diets were imposed with an energy reduction of 70 Kcal (NC1 and NC2 for SBM and CM respectively). All dietary treatments received a background application of phytase (200 ppm Ronozyme Hi-Phos, Ca and avP matrix applied), xylanase (200 ppm Ronozyme WX, 60 Kcal matrix applied) and protease (200 ppm Ronozyme ProAct, 3-4% CP and AAs matrix applied). To test the effects of NSP-multienzyme combinations, the NC diets were supplemented with one of two commercial enzyme products (Ronozyme VP enzyme, VP, at 250 ppm alone; or VP combined with Ronozyme Multigrain enzyme, MG, at 100 ppm). There were seven replicate pens of 15 birds per treatment. Dietary treatments were fed for the starter (1-10 d), grower (11-24 d) and finisher (25-35 d) phases.

Table 1 - Effect of NSP-multienzyme on broiler chicken performance at 35 days of age.

	Soybean Meal				Canola Meal					
1-35 d	PC1	NC1	NC1 + VP	NC1+ VP+MG	PC2	NC2	NC2 + VP	NC2+ VP+MG	SEM	P-*value*
FI (g/b)	3562^{b}	3683^{a}	3586^{ab}	3581^{ab}	3539^{b}	3638^{a}	3566^{ab}	3564^{b}	24.14	0.002
BWG (g/b)	2488^{abc}	2438^{c}	2446^{bc}	2456^{bc}	2546^{a}	2490^{abc}	2501^{abc}	2518^{ab}	14.65	0.001
FCR (g/g)	1.432^{c}	1.510^{a}	1.466^{b}	1.458^{b}	1.390^{d}	1.461^{b}	1.425^{c}	1.415^{c}	0.005	0.001

FI- feed intake; BWG - body weight gain; FCR 1 - Feed conversion ratio; PC – positive control; NC- negative control; VP – Ronozyme VP enzyme; MG – Ronozyme multigrain enzyme; SEM – standard error of the mean

Table 1 illustrates that a 70 Kcal reduction imposed to the NC dietary treatments significantly affected ($P<0.05$) bird performance, increasing FI and FCR compared to the PC diets. Birds fed the NC diets with NSP-multienzyme treatments presented a FI statistically similar to the PC treatment. For both protein sources, application of VP alone or with MG improved the FCR significantly compared to the NC diets ($P<0.05$). Dietary treatments had no statistically significant impact on BWG within each protein meal. In conclusion, the NC diets increased FCR by approximately 8.0 and 7.0 points in NC-SBM and NC-CM diets, respectively. This deteriorated FCR was partly recovered by adding VP (4.4 and 3.6 points) and VP + MG (5.2 and 4.6 points) in SBM and CM diets, respectively.

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CHEMICAL PROFILE AND EFFECTS OF MODERN AUSTRALIAN SORGHUM POLYPHENOLIC-RICH EXTRACTS ON FEED PHYTASE AND PROTEASE ACTIVITY

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Summary

While the beneficial roles of feed enzymes for poultry are well-established both in increasing nutrient bioavailability and reducing the impact of anti-nutritional factors (ANFs), their possible interactions with polyphenols are unknown. The purpose of the current work was to investigate the chemical composition of polyphenol-rich extracts from Australian sorghum (Liberty, Cracka, Buster) and tentatively identify compounds in a complex mixture. These extracts were then tested as inhibitors of two poultry feed enzymes, phytase and serine protease. Effects were measured through the novel use and interpretation of isothermal titration calorimetry (ITC) and a colourimetric, kinetic assay.

I. INTRODUCTION

While the inclusion of feed enzymes is routine in poultry fed sorghum-based diets, the effects of these enzymes are often muted or substandard, especially with phytase (Selle et al., 2018). The exact mechanism for this underperformance is not known, however, it is most likely caused by one or all of three key endogenous grain components: kafirin, phytate and phenolic compounds. Phenolic compounds, routinely identified in sorghum, are known to be antinutritional, especially with regard to animal nutrition (Velickovic and Stanic-Vucinic, 2018). This antinutritional effect comes through precipitation of macromolecules thus limiting digestibility, interactions with the complex grain matrix and interference with digestive enzymes. Higher molecular weight compounds such as condensed and hydrolyzable tannins are thought to be one of the culprits of these effects (Bravo, 1998).

Feed manufacturers must take these potential interactions into account when preparing grain and formulating feed mixtures to include exogenous enzymes. Modern Australian varieties have been bred to reduce tannin content and are, for the most part, considered to be tannin-free (Selle et al., 2018). While ‘tannins’ in the traditional sense may be significantly reduced in modern varieties, ‘non-tannin’ phenolics are very much still present and have the potential to produce anti-nutritional effects (Liu et al., 2015). These phenolics along with kafirin and phytate may be interacting in complex ways that might reduce the effectiveness of feed enzymes, overall digestibility and energy utilisation. Therefore, a thorough analysis of the complex matrix and its components can lead to better understanding of the grain’s role in animal feed and ways to increase its performance and profitability.

II. METHOD

MR-Buster (Buster), Cracka and Liberty sorghum were provided by DSM Nutritional Products and harvested in 2017 in Queensland. Phytase and serine protease feed enzymes were also provided by DSM. Sorghum grain was defatted and extracted for polyphenols using 70% aqueous acetone following Harbertson et al. (2014). The Folin-Ciocalteu (F-C) method, following Ainsworth and Gillespie (2007), was used to determine the total phenolic content

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(TPC) of the polyphenol-rich extract. Commercial extracts were kindly provided by Silvateam (Italy). FT-IR analysis was performed using a diamond ATR crystal between the wavenumbers 4000 and 400 cm^{-1}. Mass spectrometry was performed on a Waters Synapt G2-Si MALDI-ToF and ESI-ToF mass spectrometer. The effect of sorghum polyphenol-rich extracts on phytase activity was determined through ITC. ITC analysis was conducted using a TA Analysis NanoITC (TA Instruments, New Castle, DE). The injection syringe contained 20 mM phytate and was titrated into a mixture of sorghum polyphenol-rich extract and phytase over two injections, two and five µL, at 30℃, pH 5.0 ± 0.2 and 285 rpm stirring speed. The sample cell contained phytase, 4.075 FYT/mL, alone or with a range of sorghum polyphenol-rich extracts. The effect of sorghum polyphenol-rich extracts on serine protease activity was determined by colourimetric enzyme activity assay using a small, synthetic substrate.

III. RESULTS

Twenty grams of defatted sorghum were extracted with 70% aqueous acetone and freeze-dried. Table 1 shows the extracts quantified as grams of polyphenol extract per kilogram of grain (g/kg) and as TPC in milligrams gallic acid equivalent per gram of extract (mg GAE/g). Liberty was found to have a significantly ($P < 0.001$) lower TPC than both red sorghums, Buster and Cracka.

Table 1 - Quantification of sorghum polyphenol-rich extracts.

	Buster	Cracka	Liberty
Color	Red	Red	White
Amount of extract (g/kg)	4.02 ± 1.05 (n = 3)	4.75 ± 0.84 (n = 6)	3.52 ± 1.00 (n = 6)
TPC (mg GAE/g)	8.69 ± 2.99 (n = 24)	7.99 ± 1.33 (n = 21)	3.53 ± 0.79 (n = 27)

Values are ± 1 standard deviation

FT-IR analysis indicated the spectra for sorghum extracts matched closely to each other and shared similar features to two commercial extracts, quebracho and grape seed, known to contain tannins (Figure 1). Analysis by MALDI-ToF-MS allowed for the clear separation between red and white sorghum through principal components analysis (PCA) (Figure 2). ESI-MS^2 provided tentative identifications of compounds present in the extracts to find primarily fatty acids, polyphenols and lignin-like compounds, including caffeoyl, feruloyl and coumaroyl glycerol esters.

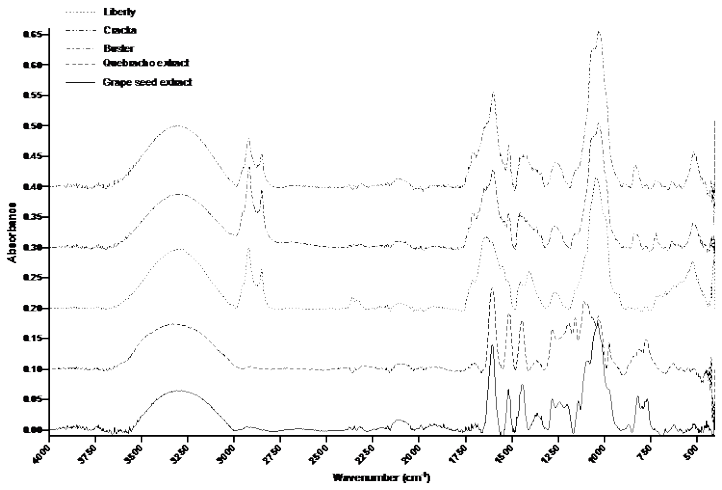

Figure 1 - FT-IR spectra of sorghum polyphenol-rich extracts and commercial extracts.

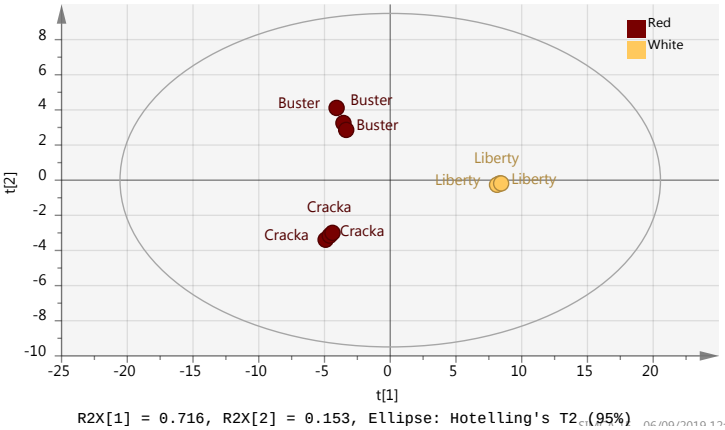

Figure 2 - PCA plot from MALDI-ToF-MS of sorghum polyphenol-rich extracts.

The presence of sorghum polyphenol-rich extracts inhibited phytase activity up to 100% in the ITC *in vitro* model (Figure 3) whereas serine protease inhibition was limited to 20-30% (Figure 4). Liberty and Cracka inhibited phytase the most. Inhibition of the serine protease was found to be mixed non-competitive.

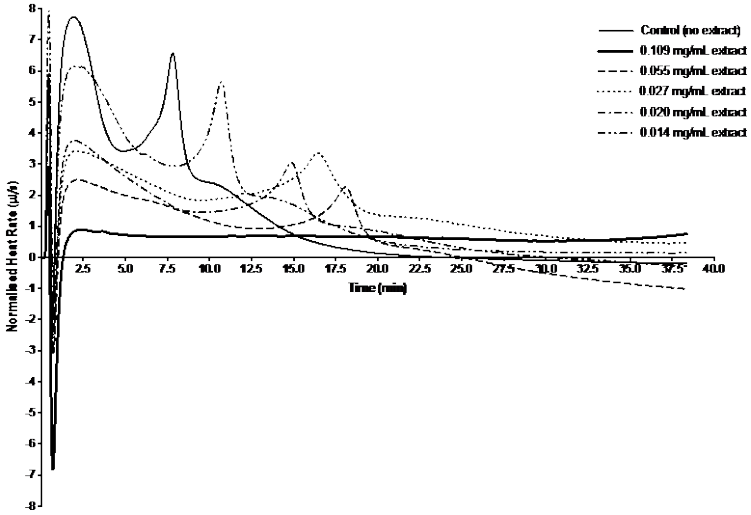

Figure 3 - ITC monitoring of phytase inhibition by Liberty extract.

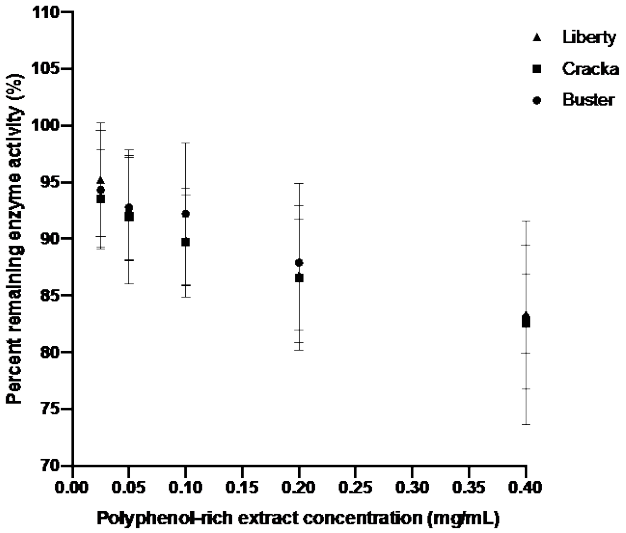

Figure 4 – Percent inhibition of serine protease activity by sorghum extracts

IV. DISCUSSION

Polyphenol-rich extracts prepared from three Australian sorghums (Liberty, Buster and Cracka) were found to contain low to intermediate levels of phenolics and tentatively identified lignin-like derivatives, often associated with cross-linking polysaccharides in the cell wall matrix (Hatfield et al., 2017). Taylor (2005) has suggested these types of phenolics may hinder normal digestion. Further analysis of the polyphenol-rich extract including LC-MS is still needed to isolate specific compounds of interest. Phytase proved much more susceptible to inhibition than the serine protease and was inhibited most by Liberty and Cracka. This inhibition may explain the muted responses often seen in sorghum diets dosed with phytase (Selle et al., 2018). It is possible that in *in vivo* conditions phenolic compounds in sorghum may only partially inhibit phytase activity which could contribute to variance in digestible phosphorus yield, especially when low phytase inclusion concentrations are used. In addition to direct enzyme inhibition, phenolics may interact with phytate *in vivo* either directly or indirectly through phytate-starch/kafirin complexes. Phenolics and phytate have been found to positively correlate, most likely due to their proximity in the aleurone layer (Selle et al., 2018). Effects under more commercial conditions still need to be investigated and whether these *in vitro* responses can be replicated *in vivo* is uncertain. Serine protease, on the other hand, was least inhibited by Liberty and most by Buster. Inhibition values of the serine protease approached 30% indicating that even at high levels of polyphenol inclusion the enzyme remained robust and maintained sufficient activity.

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PRODUCTION PERFORMANCE AND FLOCK UNIFORMITY OF BROILERS SUPPLEMENTED WITH AN EXOGENOUS PROTEASE

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Poultry rely on enzymatic digestion more so than any other livestock species due to their very short digesta transit time and because their large intestines lack the bacteria that aids other species in digestion. Because of this, their ability to digest nutrients in the feed is not absolute. Recently, along with phytase and non-starch polysaccharide-degrading enzymes, protease has become a standard component in broiler diets to improve nutrient digestibility and utilization. However, there are several other known mechanisms which support the observed benefits of protease in broiler nutrition. These include improvements in energy digestibility, degradation of protein-based anti-nutrients, enhancement of gut physiology, improvement in intestinal integrity, and beneficial effects on gut microbiota (Ghazi et al., 2002; Wu et al., 2014). Singly or collectively, these mechanisms may impart positive impact on animal health and performance. To this end, a study was conducted to evaluate the effects of protease supplementation on animal performance and flock uniformity in broiler diets.

A total of 960-d-old male Cobb chickens were randomly assigned to receive 1 of 4 dietary treatments: 1) Treatment 1, a standard diet based on corn and soybean meal (T1), 2) Treatment 2, same as T1 but reduced by 2.5% digestible AA and 20 kcal/kg ME + 0.0125% Protease (T2), 3) Treatment 3, same as T1 but reduced by 5.0% digestible AA and 20 kcal/kg ME (T3), 4) Treatment 4, same as T3 + 0.0125% Protease (T4). A completely randomized design consisting of 4 treatments, 12 replicate pens, and 20 birds in each pen was used. Flock uniformity was measured by weighing birds individually at the start (d 0) and at the end of the study (d 42) and was expressed as coefficient of variation (CV) in live weight, with increased CV values synonymous with decreased uniformity. Relative to T1 and T3, protease supplementation in T2 and T4 diets increased ($P < 0.05$) body weight gain (BWG) (2.453 vs. 2.509 and 2.365 vs. 2.422 kg) and decreased ($P < 0.05$) FCR (1.758 vs. 1.736 and 1.799 vs. 1.770) at d 42, respectively. Compared with T1, reducing the levels of digestible AA and ME in T3 adversely affected BWG and FCR at d 42; however, protease supplementation in T4 diet allowed birds to restore performance that was not significantly different to those birds fed with T1 (standard diet). There was no significant difference among the treatment groups in terms of mortality. Flock uniformity was significantly improved ($P < 0.05$) by protease supplementation in T2 diet relative to T1 (4.85 vs. 7.65) but had no effect in birds fed with T3 and T4 diets (8.55 vs 6.78). There was no difference in flock uniformity between birds fed T1, T3, and T4 diets. Since flock uniformity is known to be influenced by the concentration of AA in broiler diets, the 5% reduction in digestible AA might have been too much relative to the level of AA uplift that the protease can provide, hence, no significant effect was observed between T3 and T4. Overall, protease supplementation may improve or restore losses in animal performance when used in nutrient reduced broiler diets. In addition, it may also help promote better flock uniformity, thus, reducing potential economic losses due to increased number of downgrades at harvest.

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EFFECTS OF SUPPLEMENTAL PROTEASE AND DIET TYPE ON ENERGY UTILISATION AND NUTRIENT DIGESTIBILTY OF BROILERS FROM 9 TO 22 DAYS OF AGE

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Dietary protease supplementation has been observed to improve broiler growth performance and amino acid digestibility (Angel et al., 2011; Fru-Nji et al., 2011). Improvements in broiler energy utilisation have also been observed with supplemental protease (Sorbara, 2009; Freitas et al., 2011). These positive extra-proteinaceous effects may be influenced by age and diet type. In addition, these effects may vary throughout the broiler small intestine. Therefore, an experiment was conducted to evaluate the effects of supplemental protease and diet type on apparent metabolizable energy (AME) and nutrient digestibility in broilers from 9 to 22 d of age.

At hatch, a total of 400 Cobb 500 mixed-sex broilers were obtained from a local hatchery, distributed into 4 large floor pens, and were offered 1 of 4 dietary treatments. At 6 d of age, 336 birds (84 from each of the 4 treatment groups) were weighed and distributed into 56 battery cages (6 birds per cage; 14 replicates per treatment) in a randomised complete block design. Dietary treatments consisted of a 2 × 2 factorial arrangement with diet type (corn- or wheat-based) and protease inclusion (0 or 200 mg/kg) as the main factors. Feed was provided in 2 phases; starter (1 to 14 d of age; crumbles) and grower (15 to 22 d of age; pellets). All diets were formulated to contain phytase (supplemented at 200 mg/kg) and xylanase (supplemented at 200 mg/kg). Excreta and feed intake were collected and recorded from 9 to 11 and 19 to 21 d of age for AME determination, total-tract N and starch digestibility. At 22 d of age, jejunal and ileal digesta contents from 4 birds per cage were collected, pooled, and analysed for Ti, N, starch, and gross energy concentrations for apparent N and starch digestibility and digestible energy (DE).

Diet type influenced ($P < 0.05$) total-tract N and starch digestibility, and AME from 9 to 11 d of age; and N digestibility and AME from 19 to 21 d of age. Broilers offered corn-based diets had higher total-tract N and starch digestibility coefficients and AME than those offered wheat-based diets. The effect of protease was greater ($P < 0.05$) for jejunal (N, starch, DE) and ileal (N and DE) digestibility in the corn-based diet compared with the wheat-based diet, resulting in a diet type by protease inclusion interaction. Birds offered the wheat-based diets with and without protease and the corn-based diet with protease exhibited a higher jejunal and ileal N digestibility than those offered the corn-based diet without protease. Additionally, broilers offered the corn-based diet with protease exhibited the highest starch digestibility coefficient (jejunal) and DE (jejunal and ileal) of all treatments. A main effect of protease inclusion ($P < 0.05$) was also observed to affect ileal starch digestibility; broilers offered diets with protease had a higher digestibility coefficient than those without protease.

These results indicated that both energy utilisation and nutrient digestibility of broilers may be influenced by diet type and protease supplementation. Additional research is warranted to further quantify these effects and the modes of action responsible for these responses.

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GROWTH PERFORMANCE OF BROILER CHICKENS OFFERED MAIZE- VERSUS WHEAT-BASED, REDUCED CRUDE PROTEIN DIETS

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Summary

Either maize- or wheat-based diets formulated to contain dietary crude protein (CP) levels of 222, 193 and 165 g/kg were offered to male broiler chickens from 7 to 35 days post-hatch as a 2 x 3 factorial array of dietary treatments. The weight gains of birds offered the 193 and 165 g/kg CP maize-based diets were significantly greater than the 222 g/kg CP diet by 8.22% and 7.05%, respectively. The transition from 222 to 165 g/kg CP diets generated a feed intake increase of 8.51% with maize-based diets but a feed intake decrease of 18.5% with wheat-based diets. Growth performance of birds offered the 165 g/kg CP wheat-based diet was remarkably inferior and the outcomes and implications of this maize versus wheat comparison are discussed

I. INTRODUCTION

Global chicken-meat production has been predicted to increase considerably between 2005 and 2050 (Alexandratos and Bruisma, 2012) and this demands sustainable practices. Reducing dietary crude protein (CP) substantially without compromising broiler performance has the potential to provide tangible reductions in nitrogen excretion and environmental pollution, with improved bird welfare outcomes and economic benefits (Kidd and Choct, 2017). Substantial reductions in dietary CP for broilers will reduce requirements for soybean meal particularly. However, substantial CP reductions usually compromise growth performance in association with increased fat deposition. Several reasons have been suggested for the poor performance of broilers offered substantially reduced CP diets that include a variety of both amino acid (AA) and non-AA acid limitations (Waldroup, 2017; Siegert et al., 2015). Nevertheless, the impact of the feed grain used in reduced CP diets on broiler performance has received little attention. Thus, the purpose of this study was to compare maize- and wheat-based diets on broiler performance in the context of substantial reductions in dietary CP.

II. METHODOLOGY

A total of 216 off-sex male Ross 308 broilers were offered either maize- or wheat-based, iso-energetic (12.85 MJ/kg) diets, steam-pelleted at a conditioning temperature of 80°C from 7 to 35 days post-hatch. The diets were formulated to contain 222, 193 and 165 g/kg CP as shown in Table 1. All diets were formulated to standardised ileal digestible lysine level of 11.50 g/kg, glycine equivalents of 14.51 g/kg and the dietary electrolyte balance was maintained at 250 mEq/kg. Each diet was offered to 6 replicate cages (6 birds per cage) as a 2 x 3 factorial array of dietary treatments. Weight gains, feed intakes and feed conversion ratios (FCR) were determined from 7 to 35 days post-hatch as were relative abdominal fat-pad weights. Experimental data were analysed via the SPSS Statistics 24 program (IBM Corporation. Somers, NY). The feeding study fully complied with specific guidelines (2016/973) approved by the Animal Ethics Committee of the University of Sydney.

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III. RESULTS

Significant treatment interactions ($P < 0.001$) were observed for growth performance parameters as shown in Table 2. These interactions were driven by the extremely poor performance of birds offered the 165 g/kg CP wheat-based diets. Weight gain was inferior by 34.6% (1549 versus 2370 g/bird), feed intake by 18.3% (2843 versus 3481 g/bird) and FCR by 24.9% (1.840 versus 1.473) in comparison to their 165 g/kg CP maize-based diet counterparts. Birds offered the 165 g/kg CP maize-based diet had higher weight gains by 7.05% (2370 versus 2214 g/bird; $P = 0.049$) than the corresponding 222 g/kg CP diet. Similarly, the 193 g/kg CP maize-based diet supported a weight gain advantage of 8.22% (2396 versus 2214 g/bird; $P = 0.023$) where the significant P values are based on pair-wise comparisons. Instructively, with the transition from 222 to 165 g/kg CP diets, maize-based diets generated a feed intake increase of 8.51% (3481 versus 3208 g/bird; $P = 0.004$); in contrast, wheat-based diets triggered a decline in feed intake of 18.5% (2843 versus 3487 g/bird; $P < 0.001$). However, maize-based diets increased relative fat-pad weights by a two-fold factor (12.77 versus 6.42 g/bird) but wheat-based diets did not influence fat deposition.

IV. DISCUSSION

The growth performance of the majority of broiler chickens comfortably exceeded 2019 Ross 308 objectives in the present study but with the obvious exception of birds offered the 165 g/kg CP wheat-based diets as their performance was remarkably inferior. However, there is a precedent for this as equally poor performance of birds offered reduced CP wheat-based diets were observed in another of our studies (as yet unpublished data). The underlying reasons for these unexpected outcomes are obscure but it does appear that maize-based diets are more conducive to reductions in CP than wheat-based diets. However, it is imperative that the underlying causative factors are identified.

Substantial differences in broiler growth performance were observed between maize and wheat-based diets when dietary CP was reduced to 165 g/kg where feed grain inclusions were 721 and 751 g/kg, respectively. The relevance of starch and protein digestive dynamics in reduced-CP diets have been considered by Liu and Selle (2017) and in this context it is noteworthy that wheat starch digestion rates are more rapid than maize as demonstrated under both in vivo (Liu et al., 2019) and in vitro (Giuberti et al., 2012) conditions. In reduced CP diets, more slowly digestible maize starch may spare AA from catabolism in the gut mucosa, thereby enhancing their post-enteral availability. In contrast, more rapidly digestible wheat starch may increase AA catabolism in enterocytes along the posterior small intestine. Also, rapidly digestible wheat starch may flood the anterior small intestine with glucose to the extent that AA and glucose are competing for intestinal uptakes via co-absorption with sodium through their respective Na^+-dependent transport systems. This is supported by Moss et al. (2018) who reported significant negative correlations between proximal ileal glucose and AA digestibility coefficients in 12 of the 16 AA assessed.

The protein content of wheat is greater than maize, consequently there are more unbound AA in a reduced CP, wheat-based diets because soybean meal inclusions are less in wheat-based diets. The 165 g/kg CP diets contained either 113 g/kg soybean meal with maize but only 48 g/kg soybean meal with wheat-based diets. Consequently, wheat contained 49.4 g/kg unbound AA as opposed to 38.5 g/kg for maize when the 165 g/kg CP diets are compared in the present study as shown in Table 1.

Table 1 - Composition of experimental diets.

Feed ingredient (g/kg)	Diet 1A	Diet 2B	Diet 3C	Diet 4D	Diet 5E	Diet 6F
Wheat (107)	-	-	-	525	637	751
Maize (81)	511	615	721	-	-	-
Canola seed	60	60	60	60	60	60
Soybean meal (483)	334	228	113	300	177	48
Soy oil	35	18	-	52	36	20
L-lysine	1.60	4.69	8.12	2.36	5.93	9.72
D,L-methionine	2.67	3.54	4.53	2.75	3.74	4.81
L-threonine	1.18	2.56	4.10	1.59	3.21	4.93
L-tryptophan	-	0.15	0.79	-	0.02	0.67
L-valine	1.80	1.93	3.88	0.47	2.47	4.61
L-arginine	-	2.45	5.77	-	3.36	6.99
L-isoleucine	-	1.50	3.46	0.01	2.01	4.15
L-leucine	-		1.41	-	1.91	5.39
L-histidine	-		0.81	-	0.37	1.55
Glycine	0.32	1.86	3.57	0.41	2.12	3.95
L-serine	0.01	1.82	3.84	0.43	2.52	4.76
Sodium chloride	3.77	-	0.53	2.23	-	-
Sodium bicarbonate	0.89	6.43	5.72	2.90	6.15	6.16
Potassium carbonate	-	0.55	6.69	-	3.25	9.49
Limestone	5.96	5.90	5.82	5.92	5.84	5.74
Dicalcium phosphate	21.2	22.7	24.4	21.6	23.3	25.1
Choline chloride	0.90	0.90	0.90	0.90	0.90	0.90
Celite	20.0	20.0	20.0	20.0	20.0	20.0
Vitamin-mineral premix	2.0	2.0	2.0	2.0	2.0	2.0
Total unbound amino acids	7.23	19.47	38.49	7.50	26.36	49.39

Table 2 - Effects of dietary treatments on growth performance from 7 to 35 days post-hatch and relative abdominal fat-pad weights.

Treatment		Growth performance			Relative
Feed Grain	CP (g/kg)	Weight gain (g/bird)	Feed intake (g/bird)	FCR (g/g)	Fat-pad weight (g/kg)
Maize	222	2214^{b}	3208^{b}	1.453^{a}	6.42^{a}
	193	2396^{c}	3386^{bc}	1.415^{a}	11.18^{b}
	165	2370^{c}	3481^{c}	1.473^{a}	12.77^{b}
Wheat	222	2403^{c}	3487^{c}	1.453^{a}	6.34^{a}
	193	2386^{c}	3507^{c}	1.471^{a}	8.45^{a}
	165	1549^{a}	2843^{a}	1.840^{b}	7.53^{a}
SEM		53.64	62.18	0.0365	0.941
Main effects:					
Feed grain					
Maize		2327	3358	1.447	10.10
Wheat		2112	3279	1.558	7.46
Crude protein					
222		2309	3347	1.453	6.40
193		2391	3447	1.443	9.78
165		1959	3162	1.656	10.15
Significance (P =)					
Feed grain		< 0.001	0.129	< 0.001	0.002
Crude protein		< 0.001	< 0.001	< 0.001	0.001
Grain × protein interaction		< 0.001	< 0.001	< 0.001	0.033

[abcde] Means within columns not sharing a common superscript are significantly different at the 5% level of probability
Mean performance: Weight gain 2220 g/bird, Intake 3319, g/bird FCR 1.518, Overall mortality rate, 4.02%

This 28.3% increase in unbound AA may disadvantage wheat-based diets due to greater imbalances of AA at the sites of protein synthesis (Selle et al., 2019). Surplus AA undergo deamination which generates ammonia that requires detoxification as excessive plasma ammonia concentrations have been associated with depressed feed intakes in rats (Noda and Chikamori, 1976). Moreover, increasing systemic ammonia levels have been associated with depressions in weight gains, feed intakes and feed efficiencies (Namroud et al., 2008) and inferior feed conversion ratios (Ospina-Rojas et al., 2014) in broiler chickens offered reduced CP diets. Thus, it is tempting to speculate that the grossly inferior performance of birds offered 165 g/kg CP wheat-based diets may have been at least partially due to excessive plasma ammonia levels and further studies would be required to confirm this. Furthermore, the wheat-based 165 g/kg CP diet contained 3.82 times more unbound supplemental L-leucine compared with the equivalent maize-based diet and excessive free leucine has been reported to reduce both valine and isoleucine availability producing negative feedback on feed intake, resulting in inferior broiler growth performance, through branched-chain AA antagonisms (Smith and Austic, 1978; Burnham et al., 1992).

In conclusion, this study would suggest that substantial CP reductions in wheat-based broiler diets may be better realised by more modest dietary feed grain increases in the formulation which would be facilitated by the partial substitution of soybean meal with feedstuffs containing lesser protein contents. Limiting wheat inclusions, dietary starch levels and quantities of unbound AA may permit lower CP thresholds to be achieved without compromised growth performance and increased fat deposition.

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THE INFLUENCE OF FEED GRAINS IN BROILER DIETS: WHEAT VERSUS MAIZE IN THE CONTEXT OF REDUCED-CRUDE PROTEIN DIETS

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Summary

Reduced crude protein (CP) diets in broilers have been widely examined for over five decades. There has been considerable focus on the optimisation of unbound amino acid inclusion levels in improving broiler performance. Whilst this is an important factor, little research has been conducted comparing the influence of wheat with maize in reduced CP diets. Wheat and maize are two major feed grains used in intensive poultry production globally. Factors that appear to influence performance parameters of bird weight, feed intake and feed conversation ratios, stem from the compositional differences between the grains. Wheat has higher CP levels compared to maize which results in higher inclusions of unbound (crystalline or synthetic) amino acids in wheat-based diets. Wheat starch is more rapidly digested than maize starch, which may have negative impacts on intestinal uptakes of unbound amino acids. Wheat contains more soluble NSP than maize and therefore has the potential to increase gut viscosity, but this adverse effect can be addressed with exogenous enzymes. The higher unbound amino acid levels in wheat-based, reduced CP diets may increase plasma ammonia levels following deamination of unbalanced, surplus amino acids.

I. INTRODUCTION

Globally, maize and wheat are the major cereal grains used in broiler feed. Maize is predominant in Asia and the Americas, whereas wheat is in Australia, New Zealand and Europe. Extensive research has been undertaken in refining dietary strategies to optimise broiler performance when offered reduced crude protein (CP) diets. In feed, these grains have been optimized by the inclusion of feed enzymes and unbound (crystalline or synthetic) amino acids. Reduced CP diets have demonstrated beneficial outcomes in enhancing litter quality, minimising foot pad dermatitis (Dunlop et al., 2016) and reducing nitrogen emissions (Lemme et al., 2019).

Despite improvements in the formulation of reduced CP diets, poor bird performance is still observed in CP levels < 180 g/kg (Belloir et al., 2017). Underlying factors are often attributed to potential deficiencies in amino acids, including valine, isoleucine and arginine (Dozier and Kriseld, 2019). To date, comparative studies evaluating the influence of wheat and maize within the context of reduced-CP diets have not been completed.

A series of reduced-CP diet feeding studies have been completed by the Poultry Research Foundation (unpublished data). Step-wise reductions in CP from about 210 to 165g/kg have been evaluated, without the inclusion of feed enzymes. The growth performance of birds offered the lowest CP maize-based diets has been compromised but only to a limited extent. This was also the case with the first wheat-based study; however, in two subsequent studies bird performance was notably compromised. Thus, the impression was formed that maize- based diets are more conducive to reductions in CP than wheat-based diets. Therefore, the aim of this paper is to review the compositional differences between wheat and maize, and how these factors might influence broiler performance in the context of reduced-CP diets.

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II. WHEAT VS MAIZE: INFLUENCING FACTORS ON BROILER PERFORMANCE

In poultry feed, environmental and economic factors have typically dictated the use of maize and wheat on a geographical basis. However, maize has been preferred due to the perception that maize has a greater nutritional value compared to other grains (Cowieson, 2005). Cereal grain composition is variable in terms of starch, protein, fibre, oil and amino acid content, much of which is influenced by intrinsic and extrinsic factors such as growing conditions, variety type, starch structure, drying temperature and anti-nutritive factors (Cowieson, 2005). The relevant properties of maize and wheat are shown in Table 1.

Table 1 - A comparison of the properties of maize and wheat in vitro and in vivo.

Parameter	Maize	Wheat
Giuberti et al., 2012	(n= 14)	(n = 12)
Starch (g/kg)	703	614
Protein (g/kg)	81	136
Starch: protein ratio (g/kg)	8.68	4.51
Amylose (%)	31.1	29.4
Fat (g/kg)	40	16
Starch digestion rate (g/minute)	0.017	0.135
Potential starch digestibility (%)	95.0	92.8
Predicted glycaemic index	39.5	70.4
Rapid starch (%)	20.9	29.5
Slow starch (%)	52.1	61.8
Resistant starch (%)	27.1	8.6
AMINODat® 5.0, 2016	(n = 11)	(n = 15)
Arginine (g/kg)	4.3	5.5
Histidine (g/kg)	2.6	2.7
Isoleucine (g/kg)	3.2	3.9
Leucine (g/kg)	12.0	7.7
Lysine (g/kg)	2.6	3.3
Methionine (g/kg)	1.9	1.8
Phenylalanine (g/kg)	4.8	5.3
Threonine (g/kg)	3.2	3.4
Tryptophan (g/kg)	0.7	-
Valine (g/kg)	4.2	5.0
Alanine (g/kg)	7.0	4.2
Aspartic acid (g/kg)	6.0	6.0
Cysteine (g/kg)	2.1	2.7
Glutamic acid (g/kg)	17.6	32.5
Glycine (g/kg)	3.3	4.9
Proline(g/kg)	8.8	11.5
Serine (g/kg)	4.5	5.3
Tyrosine (g/kg)	-	-
Crude protein (g/kg)	118	91
Truong et al., 2016	(n = 11)	(n = 9)
Ileal digestibility coefficient	0.950	0.916
Minimum	0.873	0.790
Maximum	0.993	0.990

Interestingly, despite compositional differences, bird performance is relatively equivalent when birds are fed standard CP levels of > 220 g/kg between the two grains

(unpublished data). However, in the context of reduced CP diets, two recent studies (unpublished data) suggest that the compositional differences between wheat and maize have a direct influence on bird performance, namely in feed conversion ratio, bird weight and feed intake. These studies have shown that birds offered wheat- compared with maize-based diets performed more poorly in their feed conversion ratios, weight gain and feed intake ($P < 0.001$). In the initial study, birds were offered 180 and 162.5 g/kg CP wheat or maize-based diets, with starch capped or uncapped. Birds offered wheat-based diets had decidedly inferior performance compared with birds offered maize-based diets. In a subsequent study, reduced CP levels (165 g/kg) wheat- or maize-based diets were offered to male broilers from 7 to 35 days post-hatch. Inferior ($P < 0.001$) performance was again observed in birds offered wheat-based diets. In both studies, such observations were not anticipated, but highlight a need to understand the underlying causes.

The literature on reduced CP diets often attributes poor bird performance to amino acid deficiencies (Dozier and Kriseld, 2019). Whilst this may be the case in certain circumstances, other factors should be considered since the chemical and nutrient composition between maize and wheat differ. Of these factors, possibly three are of greatest importance. The first and most important is the levels of CP with wheat containing 51% more CP than maize (Table 1). Within the context of reduced- CP diets, wheat-based diets contain large amounts of unbound amino acids that may lead to imbalances of amino acids at sites of protein synthesis (Selle et al 2019). Amino acids in excess have been shown to impede broiler performance in reduced CP wheat-based diets, by inducing ammonia toxicity (Noda, 1975; Namroud et al., 2008). According to Noda and Chikamori (1976), blood ammonia levels regulate feed intake via the central nervous system. It is theorised that surplus amino acids undergo deamination, mostly in the liver, producing ammonia that needs to be detoxified via a condensation reaction in which ammonia and glutamic acid are converted to glutamine and excess nitrogen subsequently excreted as uric acid (Watford and Wu 2005). Higher concentrations of ammonia in the systemic plasma have been observed to depress feed intake, resulting in reduced liveweight gain and inferior feed conversion ratios (Ospina-Rojas et al, 2014; Namroud et al., 2008).

The second factor is the competition of nutrient uptake in the intestine between glucose and unbound amino acids (Moss et al., 2018). This stems from the rate of starch digestion with wheat being more rapid than maize (0.118 versus 0.08 min^{-1}; $P = 0.048$) as reported by Liu et al (2019). Furthermore, feed grain content increases in reduced-CP diets diminish the capacity of the bird to efficiently absorb nutrients (Carré, 2004) since glucose and amino acids compete for co-absorption via common Na^{+}-dependent transport systems (Vinardell, 1990). It is theorised that slowly digestible starch in maize-based diets may spare amino acids from catabolism in the gut mucosa and therefore enhance the post enteral availability of amino acids (Moss et al., 2018). In addition, according to Herwig et al. (2019), slowly digestible starch activates nutrient sensing mechanisms related to unabsorbed nutrients, such as the ileal brake, thereby enhancing the extent of digestion by increasing transit time.

The third factor is the issue of gut viscosity as a result of increased wheat inclusion. The aim of reduced CP diets is to reduce CP through minimizing the use of soybean meal, whilst increasing feed grain. Increases in wheat content have a greater negative influence compared to maize. Unlike wheat, maize has very low levels of soluble NSP (Choct,1997). Soluble NSP is an anti-nutritive factor, and is understood to increase digesta viscosity, which has been shown to hinder bird performance as it negatively impacts digestion and nutrient absorption (Choct and Annison, 1990).

III. CONCLUSION

In the context of reduced-CP diets, the higher levels of feed grain inclusion of wheat and maize have not been sufficiently considered. Composition differences between wheat and maize may influence broiler performance, by virtue of differences in crude protein and starch digestive dynamics. These factors highlight a need for further research to better understand the influence of feed grain in the context of reduced- CP diets and how that impacts on broiler performance. This will hopefully allow for implementation of enhanced dietary strategies.

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PRE-DETERMINED STARCH AND PROTEIN DIGESTION RATES ATTAIN OPTIMAL FEED CONVERSION RATIOS IN BROILER CHICKENS

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Summary

The objective of this study was to validate the relevance of starch and protein digestive dynamics in broiler chickens offered diets based on common feedstuffs with pre-determined starch and protein digestion rates. Six diets were formulated to contain six ratios of starch to protein digestion rate (1.567 - 2.453), but similar amino acid and energy densities, which were offered to broiler chickens from 7 to 35 days post-hatch. There were quadratic relationships ($P < 0.01$) between starch and protein digestion rates, and their ratios, with feed conversion ratio (FCR). A predicted optimal 1.446 FCR was attained with a starch digestion rate of 3.31 min^{-1}. An optimal FCR of 1.450 was predicted with a protein digestion rate of 2.02 min^{-1} or with a starch:protein digestion rate ratio of 1.663. Increasing protein digestion rate, or decreasing starch digestion rate, or narrowing starch and protein digestion rate ratios tended to improve feed conversion efficiency. There were no significant differences in feed intake, weight gain and FCR between the dietary treatments. The present study confirmed the importance of starch and protein digestive dynamics in practical broiler diets and demonstrated the possibility of incorporating starch and protein digestion rates into least-cost feed formulations.

I. INTRODUCTION

Both glucose and amino acids are essential for muscle protein deposition and feed conversion efficiency and total tract nitrogen retention was reported to be influenced by protein and starch digestion in broiler chickens (Liu *et al.*, 2013). Liu and Selle (2015) found that 76% of the variation in the feed-conversion ratio (FCR) could be attributed to starch and protein digestion rates in sorghum-based diets. Quadratic relationships between proximal jejunal starch to protein disappearance rate ratios with weight gain ($R = 0.849$; $P < 0.001$) and FCR ($R = 0.838$; $P < 0.001$) in broiler chickens from 15 to 28 days post-hatch were reported by Sydenham *et al.* (2017). Embracing the concept of digestive dynamics and applying it in practical diet formulations requires an understanding of variations in protein and starch digestion rates in common feedstuffs. Five studies have been completed to quantify digestion rates of starch and protein in common feed ingredients used in Australia (Liu *et al.*, 2019) and the objective of the present study was to formulate practical broiler diets based on these pre-determined values to establish the relevance of starch and protein digestion rates on feed conversion efficiency of broiler chickens.

II. MATERIALS AND METHODS

The feeding study complied with specific guidelines approved by the Animal Ethics Committee of The University of Sydney. Five studies were previously completed to quantify starch and protein digestion rates in common poultry feedstuffs. Digestion rates were quantified by fitting exponential models between apparent digestibility coefficients of starch and protein (N) in proximal jejunum, distal jejunum, proximal ileum and distal ileum with their corresponding mean retention times by using acid insoluble ash as the marker (Liu *et al.*, 2013).

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Table 1 - Diet composition and calculated nutrient specification in six completed diets (g/kg).

Diet	1	2	3	4	5	6
Wheat	411	262	200	200	200	200
Red sorghum	100	276	385	435	479	510
Canola seed	50.0	50.0	50.0	30.0	30.0	30.0
Soybean meal	243	234	272	257	213	165
Canola Meal	100	100	19	0	0	0
Soybean oil	35.6	27.2	19.2	18.0	11.3	10.0
L-Lys HCl	2.21	2.51	2.81	3.80	5.09	6.54
DL - Met	2.14	2.27	2.78	3.19	3.56	4.01
L-Thr	0.97	0.99	1.17	1.61	2.16	2.81
L-Trp	0.00	0.00	0.00	0.00	0.00	0.01
L-Val	0.10	0.04	0.22	0.73	1.39	2.19
L-Arg	0.00	0.00	0.02	0.92	2.16	3.57
L-Ile	0.05	0.00	0.00	0.40	1.06	1.85
L-His	0.00	0.00	0.00	0.00	0.00	0.32
Salt	1.91	1.61	1.66	0.47	0.00	0.00
Sodium bicarbonate	2.03	2.50	2.68	4.47	5.17	5.19
Potassium bicarbonate	0.00	0.00	0.00	0.00	1.55	3.93
Limestone	11.1	11.2	11.7	12.0	12.1	12.3
Di-calcium phosphate	7.20	7.21	8.19	8.69	9.09	9.57
Xylanase[1]	0.05	0.05	0.05	0.05	0.05	0.05
Phytase[2]	0.10	0.10	0.10	0.10	0.10	0.10
Choline chloride	0.80	0.80	0.80	0.80	0.80	0.80
Celite™	20.0	20.0	20.0	20.0	20.0	20.0
Sand	9.89	0.00	0.00	0.00	0.00	9.77
Vit-mineral Premix[3]	2.00	2.00	2.00	2.00	2.00	2.00
Calculated nutrient specifications						
AME (MJ/kg)	12.60	12.60	12.60	12.60	12.60	12.60
Crude protein	223	223	218	210	199	186
Lys[4]	11.5	11.5	11.5	11.5	11.5	11.5
Met	5.2	5.3	5.6	5.8	6.0	6.2
Met + Cys	8.5	8.5	8.5	8.5	8.5	8.5
Thr	7.7	7.7	7.7	7.7	7.7	7.7
Trp	2.6	2.5	2.5	2.3	2.1	1.9
Iso	8.1	8.1	8.1	8.1	8.1	8.1
Leu	14.6	15.4	15.9	15.4	14.5	13.3
Arg	12.5	12.2	12.0	12.0	12.0	12.0
Val	9.2	9.2	9.2	9.2	9.2	9.2
His	4.9	4.8	4.6	4.3	3.9	3.8
Gly equivalent	13.8	13.6	13.2	12.3	11.2	9.9
Ca	8.7	8.7	8.7	8.7	8.7	8.7
Avail P	4.4	4.4	4.4	4.4	4.4	4.4
DEB	250	250	250	250	250	250
Fat	82.5	76.5	63.8	53.6	47.5	46.2
Fibre	30.8	32.1	27.2	25.0	24.6	23.8
Starch digestion rate	3.419	3.129	3.169	3.394	3.590	3.728
Protein digestion rate	2.181	2.107	2.219	2.083	1.817	1.519
Starch:protein digestion rate ratio	1.567	1.485	1.428	1.629	1.976	2.453

[1]Danisco 40,000 G ; [2]Axtra PHY TPT 10,000;

[3]The vitamin-mineral premix supplied per tonne of feed: [MIU] retinol 12, cholecalciferol 5, [g] tocopherol 50, menadione 3, thiamine 3, riboflavin 9, pyridoxine 5, cobalamin 0.025, niacin 50, pantothenate 18, folate 2, biotin 0.2, copper 20, iron 40, manganese 110, cobalt 0.25, iodine 1, molybdenum 2, zinc 90, selenium 0.3;

[4]Digestible basis for all amino acids.

In the present study, six iso-energetic and iso-nitrogenous diets (12.60 MJ/kg; 11.5 g/kg dig Lys) based on wheat, red sorghum, soybean meal, canola meal and canola seed were formulated to contain six ratios of starch and protein digestion rate ranging from 1.43 to 2.45 as shown in Table 1.

Table 2 - The influence of starch and protein digestive dynamics on growth performance from 7-35 days post-hatch.

	Formulated values[1]			Feed intake	Weight gain	FCR
Diet	Starch digestion rate	Protein digestion rate	Starch:protein digestion rate ratio	(g/bird)	(g/bird)	(g/g)
1	3.419	2.181	1.567	3469	2405	1.445
2	3.129	2.107	1.485	3599	2479	1.452
3	3.169	2.219	1.428	3631	2495	1.456
4	3.394	2.083	1.629	3580	2489	1.439
5	3.590	1.817	1.976	3660	2501	1.463
6	3.728	1.519	2.453	3624	2399	1.511
SEM				59.7	31.8	0.0221
P-				0.205	0.086	0.263

[1]Starch and protein digestion rate values were generated from previous five studies

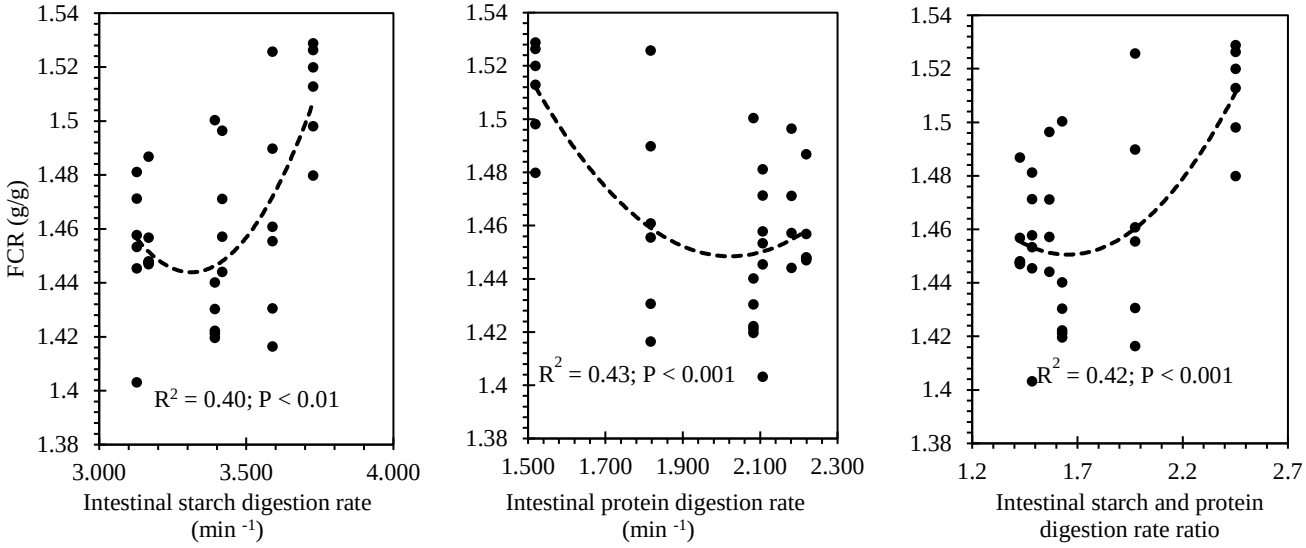

Figure 1 - The correlations between FCR and starch, protein digestion rate and starch:protein digestion rate ratios in broiler chickens from 7-35 days post-hatch.

All diets contained similar ideal protein ratios and were steam-pelleted at 80˚C and then crumbled for birds from 7 to 14 days post-hatch. The same diets were offered as pellets from 15 to 35 days post-hatch. Each of the six dietary treatments was offered to 6 replicate cages (6 birds per cage) or a total of 216 off-sex male Ross 308 chicks (parent line). Chickens had *ad libitum* access to feed and water. Initial and final body weights were determined, and feed intakes were recorded from which feed conversion ratios (FCR) were calculated. The incidence of dead or culled birds was recorded daily and their body-weights used to adjust FCR calculations. ANOVA and linear and quadratic correlation were performed using JMP® 13.0.0 and significance was determined at $P < 0.05$.

III. RESULTS AND DISCUSSION

Growth performance results are shown in Table 2 where the 4.6% mortality rate was not influenced by dietary treatments (P > 0.25). There were no significant treatment differences in feed intake, weight gain and FCR; however, there were quadratic relationships between starch and protein digestion rates, and their ratios, with FCR (Figure 1). Weight gains and feed intakes were not correlated with starch and protein digestion dynamics. The quadratic relationship (R^2 = 0.40, P = 0.003) between FCR and starch digestion rates can be described as:

$$y = 0.366x^2 - 2.423x + 5.456.$$

This indicates the optimal FCR of 1.446 was predicted when starch digestion rate equals 3.31 min^{-1}. Increasing starch digestion rate tended to compromise feed conversion efficiency or increase FCR. The quadratic relationship (R^2 = 0.43, P < 0.001), between protein digestion rates and FCR can be described as:

$$y = 0.251x^2 - 1.014x + 2.474.$$

This suggests the optimal FCR of 1.450 was predicted when protein digestion rate equals 2.02 min^{-1}. Increasing protein digestion rate tended to improve feed conversion efficiency or decrease FCR. The quadratic relationship (R^2 = 0.42, P < 0.001) between starch:protein digestion rate ratios and FCR can be described as:

$$y = 0.095x^2 - 0.316x + 1.712.$$

This shows that the optimal FCR of 1.450 was predicted when starch:protein digestion rate ratios were equal to 1.663. Narrowing the rate ratios tended to improve feed conversion efficiency or decrease FCR.

In general, starch is more rapidly digested than protein and feed conversion efficiency may be influenced by both rate and extent of starch and protein digestion (Liu and Selle, 2015). Liu *et al.* (2016) compared the influence of corn starch (rapidly digestible starch) and fish meal (rapidly digestible protein) inclusions on broiler performance and found that protein digestion rates were more influential than starch digestion rates. However, in the present study, starch and protein digestion rates were equally important in respect of FCR. Across the experimental range, retarding starch digestion rates, or accelerating protein digestion rates, or condensing starch:protein digestion rate ratios, will improve feed conversion efficiency or reduce FCR. Therefore, the present study confirmed the importance of starch and protein digestive dynamics in practical broiler diets and demonstrated the possibility of including starch and protein digestion rate data in least-cost feed formulations.

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STARCH DIGESTION ALONG THE GASTROINTESTINAL TRACT IN BROILER CHICKENS OFFERED A WHEAT- OR CORN-BASED DIET

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Starch is the most abundant source of energy in broiler diets, providing about 50 % of apparent metabolisable energy. Broiler diets generally contain approximately 40 % starch, which is thought to be completely digested within the small intestine. However, the microbiota in the large intestine also plays an important role in starch digestion, producing volatile fatty acids that act as an energy source. The potential consequence of this is an incorrect prediction of the digestible energy values of diets when considering the contribution of starch. In this regard, it is important to understand the kinetics of starch digestion and the degree of disappearance throughout the chicken gastrointestinal tract.

The present study evaluated the effects of different diet types on starch digestion along the gastrointestinal tract of broiler. Birds were offered either a wheat- or corn-based diet, with 12 replicates of six birds per treatment, raised for 35 days. Titanium dioxide was added at 5 g/kg into all diets as an indirect marker to calculate digestibility. On day 35, excreta were collected and pooled per pen, and four birds per pen were randomly euthanized to collect digesta samples from the crop, gizzard, jejunum, ileum and caeca.

The quantity of undigested starch in the gizzard was lower in birds fed the wheat-based diet compared to those fed the corn-based diet. This suggests that the gastro-duodenal reflux of digesta was comparatively greater in the anterior tract of birds fed the corn-based diet. However, the undigested starch in the jejunum was lower in birds fed the corn-based diet, indicating that the likelihood of starch reaching the hind gut for microbial fermentation was greater in birds fed the wheat-based diet. The amount of undigested starch did not change from the ileum to caeca in both diets, demonstrating that starch digestion predominantly takes place in the distal jejunum, irrespective of diet type. There was no difference between the two diets with regards to the quantity of undigested starch present in the excreta.

Table 1 - Undigested starch (g/kg dry matter intake) along the gastrointestinal tract of broilers in response to feeding diets based on either corn or wheat.

Diet	Wheat	Corn		
Starch	495.3	472.6	SEM[1]	P-value
Crop	545.9	595.4	12.35	0.060
Gizzard	331.2	1440.7	132.14	<0.001
Jejunum	103.4	78.7	5.55	0.021
Ileum	33.9	17.7	4.40	0.063
Caeca	32.8	17.6	5.07	0.141
Excreta	18.8	14.7	2.29	0.396

[1]Standard error of the mean

In conclusion, the starch from the wheat-based diet was digested more slowly than that from the corn-based diet. Furthermore, it appears that a notable amount of starch from wheat was digested by microbial fermentation in the large intestine, while the majority of starch from corn was digested earlier in the tract, with very little digested after the jejunum.

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PROTEIN DIGESTIVE DYNAMICS INFLUENCE GROWTH PERFORMANCE OF BROILER CHICKENS

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Summary

The aim of this study was to investigate protein digestive dynamics in broiler chickens using an equilateral triangle response surface design. Three apical diets were formulated to stimulate different protein digestion rates dependent on the major constituents: standard (soybean meal), rapid (whey protein) and very rapid (unbound amino acids). The three apical diets were blended at various ratios to generate a balance of seven experimental diets for the equilateral triangle design. These diets were offered to 360 off-sex male Ross 308 (parent line) broilers from 14 to 35 days post-hatch to determine growth performance. The response surface design demonstrated that maximum growth performance was achieved when birds were offered a diet containing a combination of 475 g/kg standard and 525 g/kg rapid protein digestion rates and the implications of these findings are discussed.

I. INTRODUCTION

The concept of "fast" and "slow" proteins in human nutrition was enunciated by Beaufrère et al. (2000) and consideration has been given to protein and starch digestive dynamics in poultry by Liu and Selle (2015). However, the digestion rate of protein has a greater bearing on broiler growth performance than that of starch (Liu et al. 2014). Thus, the objective of this experiment was to compare the effects of ten nutritionally equivalent diets with the same protein content (202.5 g/kg true protein) and similar amino acid profiles, but with different protein digestion rates, on broiler growth performance via an equilateral triangle response surface design. Such designs are based on three apical dietary treatments and their main protein components varied in order to generate different protein digestion rates. Soybean meal is the dominant protein source in practical broiler diets thus apical diet 1 was deemed to have a standard digestion rate in this feeding study. Whey protein is more rapidly digested than soy protein (Kuipers et al., 2007), so diet 3 was considered to have a rapid digestion rate. As unbound (crystalline or synthetic) amino acids do not require digestion they are rapidly absorbed (Wu, 2009); therefore, diet 2 was judged to have a very rapid digestion rate. The balance of seven dietary treatments are various blends of the apical diets with intermediate protein digestion rates.

II. MATERIALS AND METHODS

The feeding study complied with specific guidelines approved by the Animal Ethics Committee of The University of Sydney. Three apical diets were formulated to contain different sources of protein sources; diet 1 was based on soybean meal with minimal inclusions of unbound amino acids, diet 2 included substantial levels of unbound amino acids and diet 3 contained rapidly digestible whey protein, as shown in Table 1. The three apical diets were formulated to identical true protein content energy density with very similar levels of digestible lysine, crude protein and ideal protein ratios. The remaining seven dietary treatments were derived by blending the apical diets at various ratios as shown in Table 2. All diets were steam-pelleted at 80˚C and offered to broiler chickens from 14 to 35 days post-hatch. Each of the ten dietary treatments was offered to 6 replicate cages (6 birds per cage) or a total of 360 off-sex male Ross 308 chicks (parent line). Birds

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were allowed *ad-libitum* access to feed and water with a daily illumination period of 18 hours. Initial and final body-weights were determined, feed intakes were recorded from which feed conversion ratios (FCR) were calculated. The incidence of dead or culled birds was recorded daily and their body-weights used to adjust FCR calculations. Statistical analysis of data and generating the response surfaces was completed with R 3.0.3 software.

Table 1 - Diet composition and calculated nutrient specifications in three apical diets.

Item (g/kg)	Diet 1	Diet 2	Diet 3	Item (g/kg)	Diet 1	Diet 2	Diet 3
Diet composition				*Nutrient specifications*			
Wheat	577.10	689.00	748.04	AME(MJ/kg)	12.9	12.9	12.9
Soybean meal	323.14	161.57	-	True protein	202.5	202.5	202.5
Whey protein	-		160.56	Crude protein	222.3	203.9	214.4
Soybean oil	47.92	27.54	12.59	Starch	386.7	459.6	497.5
Limestone	11.97	12.38	12.77	Calcium	8.7	8.7	8.7
Dicalcium phosphate	6.77	8.35	10.12	Available phosphorous	4.4	4.4	4.4
Sodium chloride	3.21	-	-	Lysine	11.5	11.5	11.5
Sodium bicarbonate	-	4.71	4.76	Methionine	5.7	6.3	4.7
Potassium carbonate	-	4.40	8.07	Methionine + cysteine	8.5	8.5	8.7
l-lysine HCl	2.27	7.23	-	Threonine	7.7	7.7	8.3
d,l-methionine	3.06	4.52	1.36	Tryptophan	2.5	1.9	2.8
l-threonine	1.30	3.56	-	Isoleucine	8.1	8.1	8.6
l-tryptophan	-	0.22	-	Leucine	13.8	12.3	16.3
l-valine	0.59	3.45	0.21	Arginine	12.6	12.0	12.0
l-isoleucine	0.03	2.84	-	Valine	9.2	9.2	9.2
l-leucine	-	3.10	-	Histidine	0.5	0.4	0.4
l-arginine	-	4.10	5.74	Glycine	7.4	7.4	7.4
l-histidine	-	0.65	0.25	Serine	9.1	9.1	9.1
Glycine	-	2.39	2.70	Glycine equivalents	13.9	13.9	13.9
l-serine	-	2.91	0.41	Glutamic acid	40.48	64.09	47.85
l-glutamic acid	-	33.46	8.76	DEB (meq/kg)	242.1	240.0	240.0
Xylanase	0.10	0.10	0.10				
Phytase	0.10	0.10	0.10				
Choline chloride 60%	0.45	1.43	1.45				
Vitamin-mineral	2.00	2.00	2.00				
premix	20.00	20.00	20.00				
Celite							

Table 2 - Proportions (%) of apical diets in ten dietary treatments.

Treatment	Diet 1	Diet 2	Diet 3	Treatment	Diet 1	Diet 2	Diet 3
1	100	0	0	6	0	50	50
2	0	100	0	7	66.6	16.7	16.7
3	0	0	100	8	16.7	66.6	16.7
4	50	0	50	9	16.7	16.7	66.6
5	50	50	0	10	33.3	33.3	33.3

III. RESULTS AND DISCUSSION

Significant treatment effects ($P < 0.01$) were observed for weight gain (range 1900 to 2089 g/bird), feed intake (range2777 to 2926 g/bird) and FCR (range 1.401 to 1.484), as shown in Table 3. Thus, the best performing birds held advantages of 9.95% in weight gain, 7.96% in feed intake and 5.59% in FCR over the worst performing birds. These marked advantages may be attributed essentially to differences in protein digestion rates across the ten diets. From visualisation of the response surfaces (Figure 1), it is evident that the optimal weight gain and FCR was supported by a dietary

combination containing a combination of 475 g/kg standard (soybean meal) and 525 g/kg rapid (whey) protein digestion rates. Then the composition of the ideal diet may be deduced; this diet contains 153 g/kg soybean meal, 84 g/kg whey and a total of 13.41 g/kg of ten unbound amino acids, as shown in Table 4.

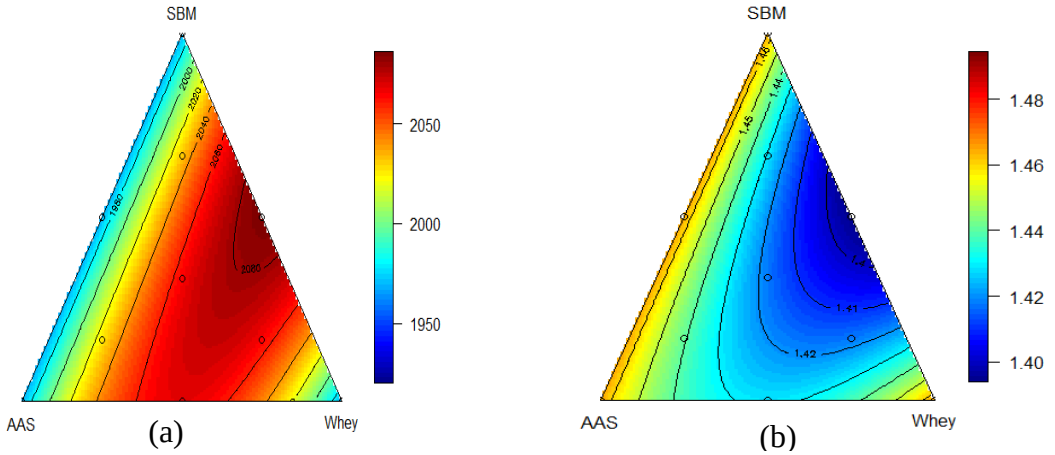

Figure 1 - Response surface of dietary treatments on weight gain (a) and FCR (b).

Table 3 - Effects of dietary treatments on growth performance.

Dietary treatment	Weight gain (g/bird)	Feed intake (g/bird)	FCR (g/g)
1	2021cd	2903bcd	1.437bc
2	1927ab	2856abc	1.484a
3	1900a	2777a	1.463ab
4	2089d	2926bcd	1.401c
5	2001bc	2946cd	1.473ab
6	2056cd	2951cd	1.446ab
7	2038cd	2936bcd	1.441bc
8	2083cd	2998d	1.440bc
9	2072cd	2931bcd	1.415c
10	2026cd	2840ab	1.403c
SEM	29.37	37.16	0.0148
Significance (P =)	0.001	0.006	0.001
LSD (P < 0.05)	83.4	105.6	0.0423

Table 4 - Composition of 'ideal' diet.

Ingredient	g/kg	Ingredient	g/kg
Wheat	666.80	*l*-valine	0.39
Soybean meal	153.50	*l*-isoleucine	0.01
Whey protein	84.30	*l*-arginine	3.01
Soybean oil	29.37	*l*-histidine	0.13
Limestone	12.40	Glycine	1.42
Dicalcium phosphate	8.53	*l*-serine	0.22
Sodium chloride	1.52	*l*-glutamic acid	4.60
Sodium bicarbonate	2.50	Xylanase	0.10
Potassium carbonate	4.24	Phytase	0.10
l-lysine HCl	1.08	Choline chloride 60%	0.98
d,l-methionine	2.17	Vitamin-mineral premix	2.00
l-threonine	0.62	Celite	20.00

abc Means within columns not sharing a common suffix are significantly different at the 5% level of probability.

Both Boirie et al. (1997) and Dangin et al. (2001) have shown that rapid protein digestion and amino acid absorption amplifies plasma amino acid levels and protein synthesis in humans. Also, rapid protein disappearance rates along the small intestine have been shown to enhance growth performance and influence the post-enteral availability of amino acids in broiler chickens (Truong et al., 2017). In this study, there was a positive linear relationship ($r = 0{\cdot}706$; $P< 0{\cdot}001$) between protein (N) disappearance rates (g/bird/day) in the distal ileum and weight gain, which illustrates the value of 'rapid protein'. These outcomes support the findings of the present study where, effectively, inclusions of 84.3 g/kg rapidly digested whey protein and certain unbound amino acids (arginine, histidine, glutamic acid, glycine, serine) in the standard wheat-soybean meal diet enhanced both weight gain and FCR. The involvement of arginine and histidine is of interest but an explanation is not straightforward. Alternatively, that unbound glutamic acid, glycine and serine may have contributed to this result is noteworthy as these "non-essential" amino acids are all involved in the elimination of excess N as uric acid via the Krebs uric acid cycle (Salway, 2018). The dietary provision of these unbound, rather than protein-bound, amino acids implies that their very rapid availability may have benefitted the relevant metabolic pathways. Diet 2 supported significantly inferior weight gain and FCR in comparison to standard diet 1 and diet 2 contained substantial unbound amino acid inclusions. This is of obvious relevance to the development of reduced-crude protein diets as they also contain substantial amounts of unbound amino acids. The 'ideal' diet shown in Table 4 would not be economically viable in practice; nevertheless, it does demonstrate that the performance of broiler chickens may be enhanced by inclusions of 'rapid' and 'very rapid' sources of protein/amino acids in a standard diet.

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UNDERSTANDING THE WOODY BREAST SYNDROME AND OTHER MYOPATHIES IN MODERN BROILER CHICKENS

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Summary

The poultry industry has seen an increase in several breast meat myopathies over the past decade. They range from woody breast syndrome (tough chicken fillet), to white striping and the so-called spaghetti meat (muscle fibre separation). All seem to be related to the quantity of connective tissue within the muscle. A number of researchers are of the opinion that they all have the same etiology. It is currently estimated that these myopathies cost the global poultry industry over $1 billion in direct and indirect costs. The incidence of these myopathies, within a given flock, appears to be related to factors such as growth rate, genetics, nutrition, management and bird activity. The resulting meat can have tougher texture, lower binding and reduced water holding, but does not present a food safety issue. However, because of the appearance of the meat some might be trimmed at the processing plant. Different segments of the industry are now working on reducing the prevalence, by modifying environmental factors and by genetic selection. The presentation will further explain the types of myopathies observed as well as the factors currently being studied in an attempt to reduce the incidence.

I. INTRODUCTION

The rise in incidence of myopathies in young broilers over the past few years has resulted in the industry looking for solutions to reduce and/or eliminate their effects in the poultry flock. The main three myopathies of concern are the so-called white striping (WS - parallel white stripes on the surface of the broiler's *pectoralis* muscle), woody breast (WB - accumulation of connective tissue fibres and fat cells in the inner *pectoralis* muscle), and spaghetti meat (SP - appearing like splitting of the muscle bundles), which seem to be associated with fast growing and heavier birds. It should be noted that other myopathies in areas such as the leg, the dorsal part of the bird, and the deep *pectoralis* muscle are detected sometimes, but not at the levels of WS or WB, and therefore currently do not cause major concern.

Estimates of the costs and causes have been reported in recent years in both scientific journals and the general press. One such example is the 2016 Wall Street Journal article titled "Bigger Chickens Bring a Tough New Problem: Woody Breast" in which it was estimated that 5-10% of commercial chicken breast products could be affected by the WB syndrome. It also suggested that the cause is not necessarily associated with the final weight of the bird but more so with how quickly the bird reaches that weight. Later that year, Kuttapan et al. (2016) estimated the annual cost to the American industry at $200 million. Today, estimates go as high as $1billion for the global poultry industry. The cost is associated with sorting out the meat, diverting some of the meat to ground and minced products, as well as trimming; i.e., downgrading of the meat. Overall, the industry has made big advancements in raising chickens faster and in a more efficient way (Zuidhof et al., 2014) and now some are suggesting that this has also resulted in increasing the incidence of myopathies (Petracci et al., 2015; Sihvo et al., 2017).

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II. DISCUSSION

Kuttappan et al. (2013), reported that the incidence of WS increases as body weight increases. They published data showing that, as broilers are raised above 2.7 kg, the incidence of severe WS increases. Today, we also know that it is related to the growth rate at a specific age of the bird, and not only to the absolute body weight. Kuttappan et al. (2013) have also looked at four different broiler strains and reported that the incidence of severe WS was 5% for the first strain, 35% for the second, 5% for the third, and 16% for the fourth strain. This shows that genetics plays a role, and currently breeding companies are working on selecting birds that are less susceptible to this myopathy. In addition, these authors reported that male birds showed higher numbers with severe WS compared to female birds (43 vs. 18%). Today, we know that overall body weight should also be taken into account, as newer data show that, at the same body weight, female birds tend to have a higher proportion of WS.

The reasons for developing these myopathies can vary quite a lot and there are different factors that can potentially contribute to their development. Overall, it appears that these myopathies are a result of muscle cell damage that does not have enough time for repair, especially in a fast-growing bird. That is one of the reasons that growth rate, at a young age (10-20 days), has been reported to be an important factor, and today some producers are using this knowledge to minimize myopathies. However, this also affects overall production efficiency which is a major driver for growers. There is also a growing body of literature focusing on oxidative stress and insufficient oxygen supply to the fast-growing pectoralis muscle, which can affect myopathy development.

White striation is the result of muscle fibre replacement with fat and connective tissue. In the past, it was more typically seen in older laying hens, but today it can be seen in young (30-50 day old) broilers. Under the microscope, these fillets also show some myodegenerative muscle fibers, inflammatory cells and infiltration of eosinophilic material (Barbut, 2019; Kuttappan et al., 2013); all are indicative of muscle cell injury and the beginning of repair. The WS fillets also result in lower water holding capacity, marinade uptake and higher cooking loss because they have less functional salt soluble proteins.

Woody breast fillets are characterized by firmer texture of the fillets. This can be detected in the live bird by palpation. When deboned fillets are placed on a flat surface, a distinct harder ridge can be observed. Sometimes, the affected fillets also show a paler appearance, some surface haemorrhages, and clear exudate on the surface. As the severity of WB and WS can vary, simple scales have been developed to rank their degree and severity (commonly a 0, 1, 2, 3 scale, Petracci et al., 2019). Sorting of the meat is important to some meat companies, when meat from flocks with a high severity of WS or WB is graded and sent to specific operations. (e.g. nugget production).

The microstructure of the WB fillets also shows the accumulation of connective tissue fibres, adipose tissue cells, and lymphocytes; the latter indicate the removal of injured muscle cells. Sihvo et al. (2017) have described this type of histology by saying, “Histological evaluation revealed a significant association of myo-degeneration and lymphocytic vasculitis with WB. Vasculitis and perivascular cell infiltration were restricted to the veins. Results indicate that WB starts focally and spreads to form a diffuse and more severe lesion… white striping often coexisted with WB”. Other researchers have hypothesized that localized hypoxia is present in WB tissue due to vascular disruption and/or stagnant tissue perfusion. When comparing the pectoral vessel density between unaffected birds and areas of focal WB in affected chickens, Sihvo et al. (2018) reported that the transverse myofibril area, per vessel, was highest in the unaffected area of muscles from cases of focal WB. This was significantly higher than in macroscopically unaffected tissue, indicating that relatively decreased blood supply may trigger the development of WB in affected birds. These authors suggested that such

changes typically originate from osmotic imbalance, for which the most likely etiologies (in developing WB), include tissue hypoxia or myo-degradation of the surrounding myofibres.

In terms of genetics, Bailey et al. (2015) compared large groups of broilers and showed that there was a difference between two purebred lines of commercial broilers with different selection history; i.e., the first group had breast meat yield of 29% and the other 21%. In both groups, there were birds with a high genetic potential for increased body weight and below average for the WB myopathies. These birds are the ones that can be used for selection, and currently that is what is being done by several breeding companies. The authors further discussed the polygenic nature of these two traits and the relatively low genetic relationships with body weight and breast meat yield, which can facilitate genetic improvement across all traits in a balanced breeding program. They also emphasized the importance of understanding the environmental and management factors that can contribute over half of the variance in WS and WB incidences.

Currently, the industry is employing different strategies to minimize the occurrence of these myopathies. They range from controlling growth rate to genetics, as well as modifying environmental factors. Bodle et al. (2018) evaluated different dietary alterations and their ability to mitigate the WS and WB syndromes in high-yielding commercial male broilers. Their test diets included a commercial reference diet: increased ratio of digestible arginine to lysine; supplementation of vitamin C; doubling the vitamin pack inclusion; reducing the digestible amino acid density of the grower phase; combination of the 4 strategies mentioned above. Overall, there was no difference in performance at the end of the starter phase; however, at the end of the grower and finisher phases, feeding lower amino acid grower diets suppressed body weight and increased feed conversion. The WB score dropped from 1.83 in the control diet to 1.49, 1.27, 1.74, 1.53, and 1.43 respectively, in the diets mentioned above, Other researchers such as Abasht et al. (2016), have also recommended using nutrition to help decrease the rate of WB and suggested vitamin C supplementation to enhance anti-oxidative metabolism.

Grading and sorting the meat is currently done by several companies, but not all. The most common way is palpitation and visual inspection; however employing near infrared (NIR) is gaining popularity (Wold et al., 2017). The detection is based on the fact that WB meat has lower protein and higher moisture content. The sensor can work at a high speed of about 200 fillets per minute, which is essential when sorting meat at a high-speed processing plant (15,000 broilers per hour, Barbut, 2015). The sensor is currently being used in several plants and fine-tuning is still going on. The goal is to have equipment with the lowest possible false positive / negative samples, as this can have a big effect.

In summary, some growers are already employing various dietary modifications, (e.g., adjusting protein content), management factors, (e.g., lighting, stacking density), as well as genetics (e.g., using breeds known for slower growth rate; lines less susceptible to these myopathies). Long-term solutions focus more on selecting birds that show fewer myopathies, and learning more about the interactions with environmental factors.

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BLACK SOLDIER FLY LARVAE IN MEAT CHICKEN DIETS MODIFIES THE FATTY ACID PROFILE IN CHICKEN BREAST MEAT

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One of the reasons chicken meat is considered a healthy food is due to its relatively low-fat level. Black soldier fly (BSF) larvae are a promising alternative feed ingredient for monogastrics, including poultry. However, the impact of feeding BSF larvae in broiler diets on the fatty acid profile of the breast chicken meat remains unknown. This study aimed to investigate the impact of up to 20% BSF larvae dietary inclusion on the fatty acid profile of meat chicken breast meat when fed until 42 days of age.

A total of 400 male day-old Ross 308 birds were assigned to 10 birds/pen, five treatment groups with eight replicates per treatment. The five dietary treatments included increasing levels of BSF larvae as follows: the starter diets (fed from day 2 -10) included 0, 2.5, 5, 7.5 and 10% BSF larvae, whereas, the grower (fed from day 11-21) and finisher diets (fed from day 22-42) included 0, 5, 10, 15 and 20% BSF larvae. All diets were isocaloric and met breeder's nutrient recommendations. At 42 days of age, two birds per treatment group were euthanised, and their breast meat immediately removed, placed in a plastic bag and stored overnight at 4°C. On the following day, ~40g of meat was weighed and kept at -20 ℃ until lyophilisation and grinding. Thereafter the fatty acid profile of the 80 chicken breasts were analysed along with lyophilised BSF larvae using a fused carbon–silica column coated with cyanopropylphenyl (BPx70, 30mx 0.25 mm id and 0.25µm thickness, SGE Analytical Science, Ring Wood, Victoria, Australia, P/N 054622) and gas chromatography according to the method described by Clayton *et al.*, 2012. A total of 48 fatty acids were identified in the breast meat samples, and data were analysed using IBM SPSS version 25 to conduct a curve estimation regression for linear, cubic, and quadratic responses. As a result, there was no significant difference among the groups in the total saturated (SFA) or total monounsaturated (MUFA) fatty acids, but individual linear increases ($P < 0.001$) were observed for SFA such as lauric acid (C12:0) and myristic acid (C14:0), as well as a linear decrease ($P < 0.001$) in stearic acid (C18:0) as the BSF larvae level increased in the diets. A significant negative correlation reflected a linear decrease of total polyunsaturated fatty acids (PUFA) in the breast meat with increasing levels of BSF. However, there were no significant changes in linoleic acid (C18:2, ω6), linolenic acid (C18:3, ω3), docosapentaenoic acid (C22:5, ω3) and docosahexaenoic acid (C22:6, ω3). There was a significant linear increase in eicosapentaenoic acid, EPA (C20:5, ω3), and mead acid (C20:3, ω9) as well as a linear decrease in adrenic acid (C22:4, ω6) with increasing inclusion of BSF. The increase of EPA and mead acid in the chicken breast meat was almost 1 and 0.5-fold, respectively, when 20% of BSF larvae was included in the diets. In conclusion, by including up to 20% BSF larvae in the diet, the concentration of total PUFA in chicken breast was reduced. However, there were significant increases (1, 0.5, and 22 fold respectively) in EPA (ω3), mead acid (ω9) and lauric acid, which are fatty acids with beneficial properties that may affect the status of chicken meat as a healthy food for humans.

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IN-OVO CORTICOSTERONE ALTERS BODY COMPOSITION IN 35 DAY OLD CHICKEN MEAT BIRDS IRRESPECTIVE OF DIETARY ARGININE CONTENT

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Summary

Increasing evidence suggests early-life exposure to maternal stress can permanently alter the development of an embryo. Such findings have significant application to the chicken meat industry due to the pedigree structure of the breeder sector, as transgenerational effects on progeny performance have been previously observed in relation to the maternal environment. Therefore, an *in-ovo* study was developed to investigate the effects of maternal stress in chicken meat birds on subsequent progeny performance traits. Additionally, dietary arginine (Arg) supplementation was implemented as a means of negating any consequences of maternal stress. Eggs were exposed to either the stress hormone corticosterone (CORT) or a control solution during embryonic development. Viable chicks were separated based on *in-ovo* treatment and fed either a control or Arg supplemented diet until 35 days of age. The findings from the current study suggest *in-ovo* exposure to CORT negatively influences the body composition of female birds by promoting fat accumulation over muscle formation, with the provision of supplementary Arg potentially alleviating these effects. Furthermore, no interactions between *in-ovo* and dietary treatments were identified in relation to body weight, body weight gain and feed conversion.

I. INTRODUCTION

Human consumption of chicken meat products has risen exponentially over the past five decades and continues to grow on a global scale. Thus chicken meat production has seen unparalleled expansion, to meet the continually increasing consumer demand (Allievi et al., 2015). Such expansion has resulted in the chicken meat industry being at the forefront of animal production, where advances in animal nutrition and genetics are near optimal. Therefore, producers are continuously looking for new and innovative methods to enhance chicken meat performance, with the maternal environment providing an economically viable method to do so. The maternal environment can be described as the overall environment a female organism encounters at the time of reproduction. Several known factors can influence the maternal environment, including maternal stress, nutrition, geographical location and individual health, ultimately altering progeny development, with permanent phenotypic effects (Reynolds et al., 2010). Previous work has shown that exposure to chronic maternal stress can negatively influence progeny performance in production animals (Reynolds et al., 2010). This is a key finding for the chicken meat industry for two primary reasons. Firstly, the chicken meat breeder industry utilises feed restriction measures in their breeder flocks. Although implemented to enhance reproductive outputs in breeder hens, an increasing body of work suggests the use of feed restriction measures induces chronic stress in breeder hens, prompted by extended periods of prolonged hunger (Zulkifli et al., 2015). Secondly, commercial chicken meat birds now spend ~40% of their life within the *in-ovo* environment and recent findings have clearly exhibited the effects of the *in-ovo* environment on embryonic development in the chicken (Ho et al., 2011). Although there is strong evidence that suggests the maternal environment does

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impact offspring performance in chicken meat birds, considerable uncertainty still remains as to the precise effects environmental variations have on progeny performance. Therefore, the aim of this study was to utilise an *in-ovo* model to investigate how early-life exposure to stress may influence performance characteristics in subsequent offspring. Additionally, dietary Arg was supplemented as a means of alleviating the negative consequences of *in-ovo* exposure to CORT. Arg has been documented to enhance protein synthesis, whilst promoting the secretion of endocrine factors such as the thyroid hormones and growth hormone, both of which are involved in growth and metabolic pathways. Thus, supplementary Arg is hypothesised to alleviate, to some extent, the phenotypic consequences derived from *in-ovo* exposure to CORT.

II. METHODOLOGY

400 eggs collected from a commercial Cobb 500 broiler breeder flock were separated into two groups, with 200 eggs receiving 1 µg of corticosterone (CORT) dissolved in absolute ethanol and the remaining 200 eggs receiving a control (CON) solution. Solutions were injected into the chorioallontoic membrane at embryonic day 11. At hatch 112 CON and 100 CORT birds were weighed, then separated into four treatment groups, (1) CORT-Control, (2) CORT-Arg, (3) CON-Arg, (4) CON-Control. Birds were provided with *ab libitum* access to both feed and water. Birds fed an Arg supplemented diet received a standard chicken meat diet + 125% Arg:Lys ratio. Individual bird body weights were recorded weekly, along with pen total feed conversion ratio. A sub-sample of birds, three per treatment (n=12) were humanely killed at day 35 and subjected to a dual-energy x-ray absorptiometry scan (DEXA) to determine total body composition. Analysis of experimental data was performed by linear mixed model analysis following the procedures of IBM®, SPSS® Statistics 25 program (Armonk, NY, USA). The data were checked for normality by the Shapiro–Wilk test. Non-normalised data was analysed using nonparametric tests including Mann-Whitney U and Kruskal-Wallis. A probability level of less than 5% ($P < 0.05$) was deemed as statistically significant.

III. RESULTS

Weight gain between day 0 and 21 did not differ between CORT or CON treated birds, nor were there any sex dependent effects in relation to *in-ovo* treatment ($P > 0.05$) (Table 1). Total weight gain from day 0 to 35 did not differ between *in-ovo* treatments; however a potential sex dependent trend appeared ($P > 0.05$). CORT treated male birds recorded superior weight gain at day 35 compared to CON treated birds. Conversely, CORT treated females recorded lower total weight gain at day 35 than CON treated birds. Additionally, CORT treated males tended to exhibit enlarged breast muscle mass (%bwt), whilst no notable variation was identified in female birds relating to breast muscle mass. Furthermore, FCR was not influenced by *in-ovo,* nor dietary treatment independently, whilst no interaction was detected between *in-ovo* and dietary treatments. Female birds fed an Arg supplemented diet tended to exhibit reduced total breast muscle yield (%bwt), whilst no interaction was observed in male birds in relation to diet ($P > 0.05$). Dietary supplementation with Arg did not significantly influence weight gain at any age ($P > 0.05$). Female birds exposed to CORT *in-ovo* exhibited greater fat mass (%bwt) and reduced total lean mass (%bwt) at 35 days of age. Conversely, CON treated female birds displayed enhanced total lean mass (%bwt) and reduced total fat mass (% bwt) (Table 2). Supplementation of Arg into the diet did not influence total bird body composition at 35 days of age, although Arg supplementation tended to enhance lean mass and reduce fat mass in CORT treated birds.

Table 1 - Effects of *in-ovo* corticosterone exposure and dietary arginine supplementation on sex dependent performance characteristics in broiler chickens from 0 – 35 days post hatch. Values are average mean ± SEM.

Treatment		Sex	Weight Gain d0 - d21 (g)	Weight Gain d0 - d35 (g)	Day 35 Breast Muscle % Bwt
In-ovo					
	CORT	Male	906.3 ± 12.58	2573.9 ± 50.61	21.2 ± 0.37
		Female	815.5 ± 10.26	1927.6 ± 29.09	20.4 ± 0.45
	CON	Male	888.2 ± 15.38	2504.4 ± 58.33	20.2 ± 0.69
		Female	820.9 ± 10.26	2013.0 ± 39.29	20.5 ± 0.48
P-value					
In-ovo x Sex			0.387	0.082	0.325
Diet					
	Arginine	Male	900.0 ± 13.58	2511.0 ± 52.37	20.9 ± 0.56
		Female	825.3 ± 9.12	2013.5 ± 28.96	19.8 ± 0.51
	Control	Male	894.2 ± 14.75	2560.1 ± 63.09	20.4 ± 0.59
		Female	811.7 ± 10.91	1945.69 ± 37.12	21.0 ± 0.36
P-value					
Diet x Sex			0.739	0.531	0.089

Table 2 - Day 35 body composition of female chicken meat birds subjected to *in-ovo* CORT or CON treatment as well as birds fed an arginine supplemented diet or control diet. Values are average mean (%bwt) ± SEM.

Treatment		BMC (%bwt)	Fat (%bwt)	Lean (%bwt)
In-ovo				
	CORT	1.14 ± .04	9.11 ± .59 [a]	88.70 ± .66 [a]
	CON	1.15 ± .04	5.71 ± .59 [b]	91.77 ± .64 [b]
P-value		*0.902*	*0.007*	*0.026*
In-ovo + diet				
	CORT control	1.13 ± 0.04	9.86 ± 1.44	87.82 ± 1.31
	CORT arginine	1.16 ± 0.04	8.36 ± 0.86	89.53 ± 0.66
	CON control	1.13 ± 0.11	4.76 ± 0.52	91.94 ± 1.31
	CON arginine	1.17 ± 0.03	6.66 ± 0.40	91.59 ± 0.59
P - value		*0.910*	*0.088*	*0.295*

[a-b] values within a column with no common superscripts differ significantly ($P < 0.05$).

IV. DISCUSSION

Although the body of work supporting feed restriction induced chronic stress in breeder hens is relatively strong, the transgenerational effects exposure to maternal stress has on subsequent performance characteristics in progeny remains unclear. The present study utilised an *in-ovo* model to investigate the potential phenotypic consequences invoked via *in-ovo* exposure to a stressor in chicken meat birds post-hatch. Previous work by Hynd et al. (2016) reported a reduction in male progeny weight at 42 days of age in birds produced from hens subjected to feed restriction measures. These findings corresponded with feed restricted hens exhibiting elevated plasma corticosterone concentrations and heterophil/lymphocyte ratios, indicating elevated levels of stress, which had been previously reported (Zulkifli et al., 2015).

Interestingly, the results from the current study suggests exposure to maternal stress may act in a sex dependent manner. However, it must be noted that significant variability exists

within the literature regarding exposure to maternal stress and its ability to disrupt offspring development. Several avian species have been exposed to maternal stress under experimental conditions, with vastly different results reported between species, as well as within species (Ahmed et al., 2016, Hayward and Wingfield, 2004). Furthermore, supplementing diets with additional Arg did not influence weight gain. Arg has been documented to promote protein synthesis as well as influence growth and metabolic pathways associated with the thyroid hormones (Ebrahimi et al., 2014) and various other growth factors in birds. However, the chickens utilised in the current study were great grandparent birds, where performance variation is greater in birds positioned higher up the breeding pedigree. The use of such birds may have therefore unintentionally 'masked' any offspring phenotypic variation influenced by the dietary and *in-ovo* treatments administered. Thus, phenotypic variation may occur in separate strains further down the breeding pedigree, as has been reported within the literature.

The findings that female birds exposed to CORT exhibited enhanced total fat mass and reduced total lean mass compared to CON birds is somewhat novel. Such variation may eventuate as a consequence of alterations to the hormonal composition of the egg, disrupting physiological processes that influence the number of myofibres developed by the embryo, which is determined embryonically (Smith, 1963). Although previous studies reported that exposure to maternal stress could 'influence' weight gain in numerous avian species, whether such variation was attributed to muscle, fat or bone mass remained elusive. Additionally, supplementing CORT exposed birds with Arg tended to reduce the phenotypic consequences associated with early-life exposure to CORT. Although male total body composition was not measured due to insufficient numbers, these findings in female birds still suggest that the variations to the maternal environment may promote undesirable carcass characteristics in chicken meat birds. However, targeting the maternal environment provides a novel approach to improve total flock uniformity and subsequent carcass characteristics, albeit in a sex-dependent manner. Thus, future studies investigating the maternal environment and its ability to alter offspring development must incorporate total carcass composition along with weight gain and FCR, with specific interest in the sex-dependent variations that predominantly occur.

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EFFECT OF HYDROXY-SELENOMETHIONINE ON PERFORMANCE OF BROILER BREEDER AND PROGENY

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Summary

This study aimed at comparing the effect of dietary supplementation with two selenium (Se) sources: hydroxy-selenomethionine (OH-SeMet) and sodium selenite (SS), on egg production, egg quality of breeder flock and growth performance of their progeny.

A total of 216 broiler breeders (AP95 Aviagen) from 56 to 65 weeks old were randomly assigned to two dietary treatments (27 replicates; 4 hens each). Hens were fed on the same basal diet with the only difference being Se sources: either SS at 0.3 ppm or OH-SeMet at 0.2 ppm. At 65 weeks of age, after artificial insemination, the eggs were collected and set for incubation, and transferred for hatching at day 18. The day old chicks were counted, and 520 mixed progeny day-old chicks were used for growth trial, in a completely randomized design in a 2 x 2 factorial: 2 sources of Se for breeders' diets and two sources of Se for progeny's diets – SS at 0.3 ppm and OH-SeMet at 0.2 ppm. The 4 treatments were replicated 13 times (10 birds each) and the birds were reared until 42 days of age.

The results showed that, for breeder hens, when compared with SS, supplementing OH-SeMet significantly increased egg production (53.8% vs. 59.4%, $P < 0.05$), Se content in the egg (0.87 µg/kg vs. 1.62 µg/kg, $P < 0.01$), eggshell strength (3.28 kg/f vs. 3.52 kg/f, $P < 0.05$) and hatchability (69.8% vs. 79.4%, $P < 0.05$). The high Se deposition in the hatching eggs benefited progeny as reflected by significantly better feed conversion ratio (-8.4 FCR points, $P < 0.05$). No significant changes were observed in feed intake and weight gain, nor interactions between maternal diets and progeny's diets.

I. INTRODUCTION

Broiler breeders require adequate levels of nutrients to reach their genetic potential in terms of productive and reproductive performance, and to transfer essential nutrients from eggs to embryo for the development of chicks (Rajashree et al., 2014).

It is known that oxidative stress impairs a breeder's performance (particularly related to aging) and embryo development. This suggests that enhanced antioxidant defenses, via effective dietary supplementation with antioxidants, can better ensure and uphold productivity, fertility and hatchability.

Among different dietary antioxidants, Selenium (Se), functioning through various selenoproteins, plays a pivotal role in the antioxidant defense system. In fact, it has been proven that Se can prevent lipid peroxidation and maintain quality of semen and ensure fertility of a breeding flock (Surai and Fisinin, 2014). Moreover, Se status in the eggs from breeding hens is of great importance for maintenance of the antioxidant system of the developing embryo, resulting in higher hatchability and quality of day-old chicks. Therefore, dietary Se supplementation is widely practiced, using either an inorganic form such as sodium selenite (SS) or organic forms like Se-yeasts or pure selenomethionine (SeMet) products.

Hydroxy-selenomethionine (OH-SeMet) is a chemically synthesized molecule and has been proven to be highly efficient in transferring Se from diet to eggs compared with SS or Se-

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yeast products, and to enhance poultry performance, particularly during critical periods of the production cycle (Jlali et al. 2014; Michiels et al., 2019; Surai et al., 2018).

The aim of this study was to compare the effect of OH-SeMet and SS on performance and egg quality of a breeder flock, and the growth performance of their progeny.

II. MATERIALS AND METHODS

A total of 216 broiler breeders (AP95 Aviagen) from 56 to 65 weeks old were randomly assigned to two dietary treatments (27 replicates; 4 hens each). Hens were fed on the same basal diet with the only difference being the Se source used: either SS at 0.3 ppm or OH-SeMet at 0.2 ppm. Egg production was recorded during two cycles of 35 days, and egg weight, egg breaking strength, Se deposition in the whole egg were determined. Fertility, hatchability, embryonic mortality and number of chicks born per hen housed were also measured. At the age of week 65, hens were inseminated using 3-cocks-semen pools (0.5 mL of fresh semen to ensure a concentration of 100×10^6 spermatozoa/mL) and repeated after 3 days and their eggs were collected for ten days after the last insemination. The eggs were incubated and transferred for hatching at day 18, the day old chicks were counted and selected and used for the growth performance trial.

A total of 520 mixed progeny chicks were allocated in a completely randomized 2 x 2 factorial design: 2 sources of Se for broiler breeders' diets and 2 sources of Se for progeny diets – SS at 0.3 ppm or OH-SeMet at 0.2 ppm (Table 1). The 4 treatments were replicated 13 times (10 birds each) and the birds were reared until 42 days. Body weight, average weight gain, average feed intake and feed conversion ratio were determined per pen for the overall experimental period.

Table 1 - Experiment: completely randomized 2 x 2 factorial design.

	Selenium supplementation			
Breeder diets	SS-0.3 ppm		OH-SeMet 0.2 ppm	
Progeny diets	SS-0.3 ppm	OH-SeMet 0.2 ppm	SS-0.3 ppm	OH-SeMet 0.2 ppm

SS = Sodium Selenite; OH-SeMet = hydroxy-selenomethionine.

For the breeder trial, data were analyzed by general linear model (GLM) using as a fixed effect the source of selenium used in the diet. For the progeny trial, data were analyzed by general linear model (GLM) in a 2 x 2 completely randomized factorial design using as fixed effects the breeder diet and the progeny diet. Means were compared by Tukey's test at a 5% probability levels (SAS, 2011).

III. RESULTS AND DISCUSSION

Table 2 shows breeder performance and egg quality. In both production cycles evaluated, dietary administration of OH-SeMet at 0.2 ppm increased egg production by 10% compared to SS at 0.3 ppm ($P = 0.038$ and $P = 0.044$, respectively). These results are in agreement with the findings of Brito et al. (2019) who showed dietary supplementation with OH-SeMet enhanced egg production in laying hens from 50 to 70 weeks of age more efficiently as compared to SS. In fact, it is well established that oxidative stress is an important factor of aging (Dunn, 2013). As the hens age, they steadily decrease productivity and eggshell quality. Compared with SS, OH-SeMet can better maintain egg production after 56 weeks of age.

Eggs from hens receiving OH-SeMet showed higher Se content ($P < 0.001$) than the SS group. This result is consistent with the finding of Jlali et al. (2013) who reported that a half dose of OH-SeMet delivers significantly higher Se level in eggs compared with SS.

Table 2 - Performance and egg quality of breeders fed on Sodium Selenite (SS) or OH-SeMet (SO).

	SS-0.3	OH-SeMet 0.2	SEM	P-value
Egg production 56-60 weeks (%)	57.13[b]	62.83[a]	1.39	0.038
Egg production 61-65 weeks (%)	50.43[b]	55.94[a]	1.38	0.044
Hatchability (%)	69.76[b]	79.39[a]	2.54	0.011
Fertility (%)	91.67	94.10	6.61	0.193
No. of chicks/hen housed*	2.15[b]	2.52[a]	0.09	0.021
Egg total Se, μg/kg, wet basis	0.874[b]	1.620[a]	0.098	<0.001
Eggshell thickness (mm)	0.409	0.416	0.002	0.152
Strength (kg/f)	3.28[b]	3.52[a]	0.050	0.007

*Chicks *per* hen = calculated by dividing the number of chicks hatched by the number of hens housed during one week.
Means within a row bearing different superscripts differ significantly (P < 0.05).

No difference was observed in eggshell thickness. However, OH-SeMet at 0.2 ppm increased eggshell strength as compared with SS (P = 0.007). This finding supports Brito et al. (2019) who reported OH-SeMet improved eggshell breaking strength, and assumed that improved eggshell quality may be related to more Se deposited in the shell and shell membrane that strengthens the shell. In fact, Se participates in the formation of the eggshell organic matrix and the highest Se concentration is detected in the shell membrane. Se level in the shell is comparable to that found in albumen (Golubkina and Papazyan, 2006).

The high content of Se in eggs of breeders receiving OH-SeMet improved hatchability of fertile eggs and the number of chicks *per* hen housed, as there was a 13.8% and 17.2% increases as compared to the SS group, respectively (P = 0.011 and P = 0.021). It is known that the incubation process causes oxidative stress. More Se deposition in the eggs from hens receiving OH-SeMet may have contributed to the increased antioxidant defense of the embryo due to higher selenoprotein expression and activity, leading to increase in hatchability (Surai and Fisinin, 2014). For fertility, no difference was observed between the two Se sources.

The growth performance (1-42 days) of the progeny trial is summarized in Table 3. No interactions (P > 0.05) were detected between the two breeder diets and progeny diets in any criteria measured. No statistical difference was found between the two Se sources for feed intake and weight gain. However, the Se sources in the breeder diet showed a significant effect on the feed conversion ratio of the progeny; the chicks from breeders receiving OH-SeMet showed significantly better FCR than the birds from breeders receiving SS (-8.4 FCR points; P = 0.017). These results suggest long-lasting maternal effects of the OH-SeMet that may be attributable to more Se deposited in the day-old chicks, leading to a better start and subsequently better performance. Similar findings have been reported by Sun et al. (2012) who observed a significant increase in body weight gain and feed efficiency in chickens from breeders fed on organic mineral diets.

Table 3 - Effects of dietary Se source for breeder and/or progeny on broiler performance (1-42 d).

	Breeder effect		Progeny effect		P-values		
	SS-0.3	OH-SeMet 0.2	SS-0.3	OH-SeMet 0.2	Breeder	Progeny	Interaction
Feed intake (g)	4711	4572	4706	4577	0.088	0.115	0.183
Weight gain (g)	2608	2654	2636	2626	0.273	0.809	0.794
Feed Conversion	1.810[b]	1.726[a]	1.789	1.747	0.017	0.217	0.178

Means within a row bearing different superscripts differ significantly (P < 0.05).

For the progeny diet effect, no statistical differences were observed between the two Se sources despite OH-SeMet-fed broilers having 4.2 FCR points lower (P = 0.217). This result

appears to be logical in that there was no major challenge to the birds during the trial. In fact, it is well established that the benefits of organic Se (namely SeMet) deposited in animals' tissue show up when birds face stressful situations. When SeMet is supplemented, more Se stored in tissues will enable birds to produce antioxidant enzymes, when feed intake drops and/or the Se requirement rises, as an effective means to ensure their metabolic needs thus growth performance.

IV. CONCLUSION

Overall, for an aged broiler breeder flock, dietary supplementation with OH-SeMet at 0.2 ppm significantly improved production, eggshell strength and hatchability, compared with SS at 0.3 ppm, which can be an effective approach to help birds maintain and prolong productive and reproductive performance when the breeder flock ages. The maternal supplementation with OH-SeMet shows a positive impact on their progeny, in terms of feed efficiency.

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FUNCTIONAL ANALYSIS OF CHANGES IN GUT MICROBIOTA GENETIC POTENTIAL IN BROILERS SUPPLEMENTED WITH 2% OREGANO

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Summary

Oregano-based products, especially oregano's most potent antimicrobial volatile compounds, carvacrol and thymol, are common ingredients of many phytobiotic products on the market. Here we investigated the changes oregano inclusion in broiler feed induces in the genetic function capabilities of the gut microbiota. Oregano changed the phylogenetic composition of the gut microbiota, resulting in a significantly reduced abundance of bacterial genes involved in bacterial motility, flagella, bacterial secretion, and bacterial ability to invade epithelial cells. Oregano may therefore reduce the genetic ability of intestinal bacteria to contribute to a range of infectious diseases. There were also reductions in the abundance of genes involved in intestinal metabolic and digestive functions.

I. INTRODUCTION

Throughout the world, poultry are a major and growing source of high-quality protein, as they outperform all other terrestrial meat production systems in water, feed, carbon, and land use efficiency. A challenge for intensive production systems has been the potential to introduce high pathogen loads and stresses on the animals. Such challenges have traditionally been managed with the assistance of antibacterial growth promoters. However, the poultry industry in Australia, and in many other regions, is working to reduce the use of in-feed antibiotics and alternative products are required to maintain health and productivity. We can look to nature for alternative antimicrobial compounds. Bacteria can infect almost all organisms, and, in response, many hosts have evolved the ability to produce antibacterial compounds to help them fight off pathogenic bacteria. Plants, especially medicinal herbs and spices, produce a range of antibacterial phytochemicals. Phytobionts such as oregano, clove and cinnamon's antimicrobial ingredients, are showing comparable, if not better, results than subclinical antibiotics in pathogen control. There are multiple reports on their effect on individual poultry pathogens such as *Salmonella*, and on performance. Lately, more reports are becoming available on their effect on whole bacterial communities. However, the changes they introduce to the functional capabilities of microbiota, i.e., what microbiota can do for the host, are equally important and give a very different picture to that produced by simple taxonomic profiling of microbiota. Here we present the first insights into the effects of oregano on the functional capabilities of intestinal microbiota in broilers.

II. METHOD

Dried oregano (Turkish, Saucy Spice Company, NSW, containing 2-3% carvacrol) was used to make a powder with an average particle diameter of 10μm. Chicken starter diet (Red Hen, Laucke Mills, Australia) with no antimicrobials or coccidiostats was used for the duration of the trial. The oregano was mixed into the feed at a 2% inclusion rate (0.02 kg/kg w/w). The study was approved by the Animal Ethics Committee of Central Queensland University under

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approval number 0000020312. One day old Ross Broiler 308 birds (Bond Enterprises, Toowoomba) were randomly distributed into two groups with n=12 per treatment. All birds were fed *ad libitum* and had unrestricted access to drinking water. Birds were individually tagged using leg bands and weighed every week for 42 days. Fresh faecal material was collected for each bird. The contents from the jejunum, ileum and caecum intestinal contents, together with faecal samples were taken for sequencing analysis.

DNA was extracted from samples using a previously described protocol (Bauer et al., 2019). The 16S rRNA gene sequencing library preparation and amplification followed the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). Sequencing was conducted on the Illumina MiSeq platform using 2x300 bp paired-end sequencing according to the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). The microbial communities of each sample were initially analysed using QIIME. Phred quality threshold had a minimum of 20. OTUs were picked at 97% similarity using UCLUST (Edgar, 2010). The PICRUSt algorithm (Langille et al., 2013) was used to predict and enumerate genetic functional categories. The sequence data are publicly available at the MG-RAST database under library accession number mgl745316 and project ID mgp89580.

III. RESULTS

We predicted the functional abilities of microbiota using the KEGG database to compare microbiota of control and 2% oregano treated birds. Adonis multivariate analysis using Jaccard distance showed significant functional differences (Adonis $P<0.05$) (Figure 1). The data show that oregano supplementation reduced the abundance of genes in functional categories involved in a number of diseases such as bladder cancer (P=5e-4), prostate cancer (P=0.045), prion diseases (P=0.007) and Shigellosis (P=2.3e-4). The microbiota from oregano supplemented birds had a lower abundance of genes involved in bile secretion (P=5e-5), bacterial motility proteins (P=0.018), flagellar assembly proteins (P=0.023), bacterial secretion (P=0.012) and bacterial invasion of epithelial cells (P=2e-4). Although there were no differences in weight throughout the lifetime of the birds, a range of metabolic functional related genes, such as carbohydrate digestion and absorption (P=1.9e-3), vitamin B6 metabolism (P=0.028), fatty acid metabolism (P=0.001), and steroid hormone biosynthesis (P=0.025) were reduced in oregano supplemented microbiota. Therefore, the data suggest that oregano changes the microbiota structure to reduce bacterial species with the genetic potential for motility, flagella and secretion and their ability to invade epithelial cells, as well as reducing functional capability to contribute to a range of diseases. However, these health beneficial functions come with the cost of reducing microbiota metabolic and digestive ability.

IV. DISCUSSION

Oregano supplementation in the current study significantly reduced the presence of genes involved in flagellar assembly, bacterial motility and bacterial invasion of epithelial cells. It was previously reported (van Alphen et al., 2012) that oregano's main antimicrobial, carvacrol, inhibited the motility of *Campylobacter jejuni* without affecting bacterial growth. This is of significance because it indicates that oregano can reduce pathogenic potential in a way that is not detectable using current culturing or microbiota sequencing methods since the bacterial numbers do not have to be affected. Sub-inhibitory concentrations of carvacrol were shown to block motility and invasion of epithelia by *C. jejuni* via interfering with flagella function (van Alphen et al., 2012); the reduction of flagellar genes in the oregano supplemented microbiota was also significant in the current study. Additionally, carvacrol has been shown to induce heat shock protein 60 and inhibit the synthesis of flagellin in *Escherichia coli* (Burt et al., 2007). Oregano essential oil also shows anti-*Giardia* activity via disruption of its adherence (Machado

et al., 2010). Moreover, oregano essential oil abolishes *Salmonella enterica* serovar Enteritidis in pre-formed biofilms on stainless steel via multi-target action mode on bacterial cell membrane (Lira et al., 2019) and is efficient against antibiotic-resistant *Salmonella enterica* (Moore-Neibel et al., 2013). The anti-*Salmonella* activity is in agreement with our functional analysis findings that oregano reduces motility, bacterial secretion, and invasion of epithelia, all major *Salmonella* weaponries.

The anticancer activity of oregano reported in our functional analysis is aligned with previously reported anticancer properties of carvacrol in breast, liver, and lung carcinomas (reviewed in Sharifi-Rad et al., 2018), coupled with strong antioxidant and anti-inflammatory capabilities (Sharifi-Rad et al., 2018).

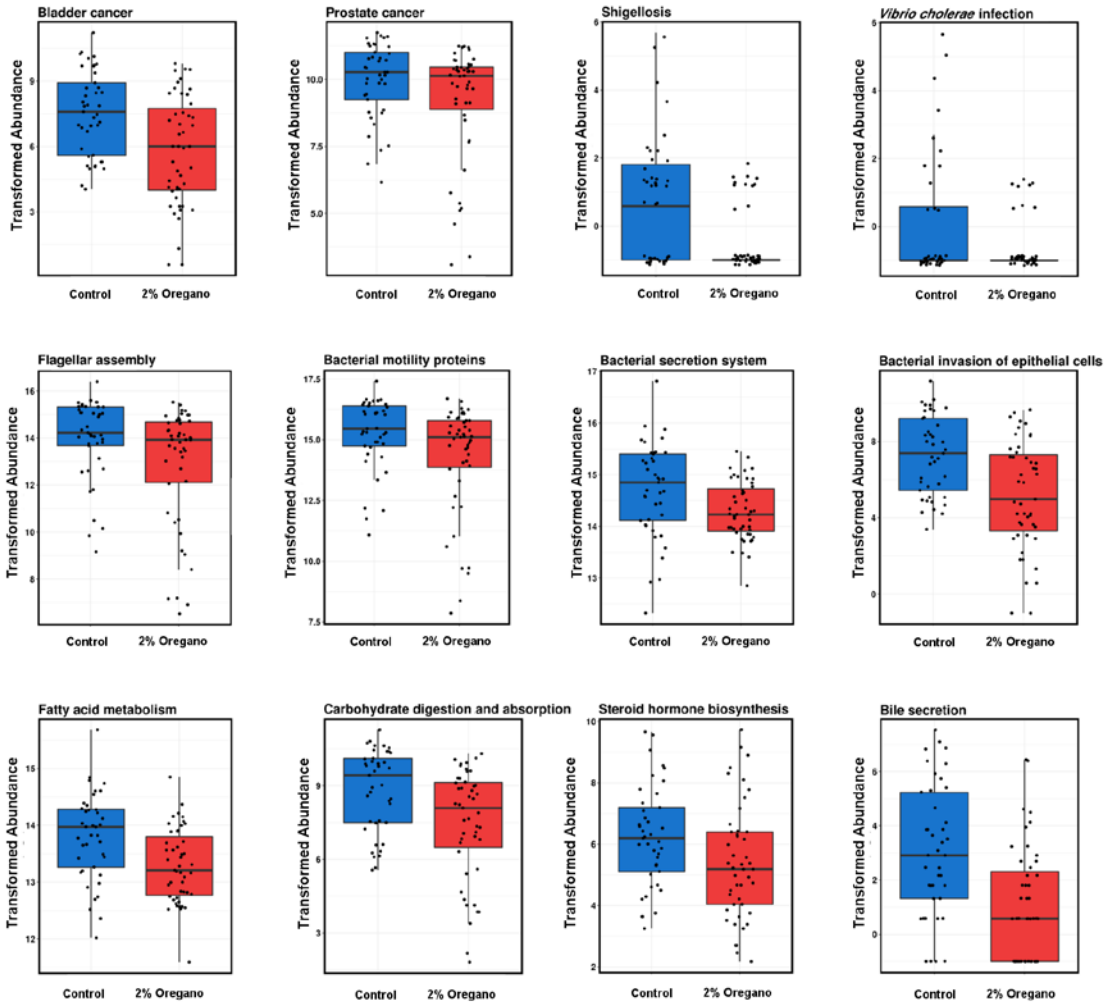

Figure 1 - Functional analysis of changed microbiota genetic potential due to influence of oregano performed in PICRUSt.

Our results indicate that oregano reduced the abundance of genes involved in digestion of carbohydrates and fats as well as steroid hormones and bile. The reports on oregano helping digestion (Reyer et al., 2017; Sharifi-Rad et al., 2018) are opposite to our findings; however, even if significantly altered, the reduction of the digestion-related genes in intestinal bacteria will not necessarily result in measurable reduction in the host digestion, but could also reduce growth of bacteria. Host digestive efficiency depends on a range of host factors, feed composition, and bacterial contribution, to name a few (Swallow, 2003). On the other hand, oregano also reduced the abundance of bacterial genes involved in host bile secretion which strengthens the possibility that oregano can indeed slow down the host digestion process. Our

study focused on genomic analysis and did not have the power to make conclusions regarding performance; however, the data on ileum histology on these same birds (not shown) demonstrated no differences in villus height or crypt depth.

Oregano in feed significantly reduced the abundance of bacterial genes involved in steroid hormone biosynthesis. Intestinal microbiota can produce biologically active molecules including sex hormones, by acquiring genes from the host via bacterial transformation. Although it is unlikely that the host depends on microbiota for steroid production, faecal transplants from male to female mice resulted in female mice producing testosterone at equivalent concentrations to that of males (Markle et al., 2013). This study was the first to show microbiota producing human host hormones. There are also other indications that oregano can induce oestrogenic responses *in vitro* and can also show oestrogen-like activity (Wielogorska et al., 2019), thus influencing steroid hormone balance. Oregano could be a suitable natural product to treat conditions related to lower oestrogen production (Beck and Hansen, 2004). The reduction in steroid synthesis capability also opens a question of possible oregano interactions with stress response via regulation of another steroid hormone – cortisone. The effects of oregano on the genetic capability of microbiota may be of higher or equal importance as the direct effect on the specific bacterial taxa abundance.

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PERFORMANCE, IMMUNITY AND BLOOD BIOCHEMICAL PARAMETERS OF BROILER CHICKENS FED DIETS CONTAINING *KAPPAPHYCUS ALVAREZII*

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Summary

A feeding trial was conducted to determine the effect of feeding red algae, *Kappaphycus alvarezii* (KPA) on the performance, immune response and blood biochemical parameters of broiler chickens. A total of 280 (7 x 5 x 8), straight run day-old broiler chicks were randomly distributed into 7 dietary treatments for 6 weeks. Seven experimental diets were formulated by adding 0% (control- T_1), 0.25% KPA (T_2), 0.50% KPA (T_3), 0.75% KPA (T_4), 1.00% KPA (T_5), 1.25% KPA (T_6) and 1.50% KPA (T_7) respectively. Body weight gain was significantly ($P<0.05$) increased in the T_7 group at 0-21 d and 0-42 d. However, feed intake (g) and feed conversion ratio (FCR) were not significantly ($P>0.05$) affected. Haem-agglutination (HA) titre and *in vivo* cell mediated immune response were significantly improved ($P<0.05$) in the T_7 group. No significant ($P>0.05$) differences were observed in total protein, creatinine and alkaline phosphatase (ALP) concentrations. However, significant ($P<0.05$) increases in albumin and aspartate aminotransferase (AST) concentration, and decreases ($P<0.05$) in alanine aminotransferase (ALT), cholesterol and uric acid concentrations occurred in T_7 compared with all other groups. The results of the present study indicate that *Kappaphycus alvarezii* (KPA) can be incorporated at 1.50% in diets for improved performance, immuno-responsiveness and blood biochemical parameters in broiler chickens.

I. INTRODUCTION

Kappaphycus alvarezii (KPA) is a red alga that is also called *Eucheuma cottonii*. Seaweeds have received significant attention for their potential as sources of natural antioxidants attributed to the carotenoids, tocopherols and polyphenols present which contribute to inhibition or suppression of free radical generation (Athukorala et al. 2006). *Kappaphycus alvarezii* is rich in enzymes, nutrients, minerals, calcium, iron, fibres and jelly forming proteins. Typical analysis of KPA shows it contains 64.2% carbohydrate, 4.5% protein, 0.9% fat, 1.07% calcium, 9.3mg/kg iron, 1520 mg/kg magnesium and 22 mg/kg niacin (Qadri et al., 2019). The total chlorophyll and carotenoid contents of KPA were 0.180 and 0.634 mg/g of fresh weight, respectively (Abirami and Kowsalya, 2011). Therefore, seaweeds are sources of nutrients as well as nutraceuticals having antioxidant, anti-mutagenic, anticoagulant, anti-cancerous and antibacterial activity and can be grown artificially and thus can be produced in aquatic farms. The objective of the present study was to evaluate the effect of *KPA* on the performance, immune-response, blood biochemical and gut health status of broiler chickens.

II. MATERIALS AND METHODS

Two hundred and eighty (280) day old straight run (sex ratio ≈ 1) chicks (CARIBRO-Vishal) were housed and distributed randomly into thirty-five groups each of 8 chicks (7 treatments × 5 replicates). The experiment was conducted strictly in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC).

The experimental diets (T_2-T_7) were supplemented with 0.25 % KPA (T_2), 0.50% KPA (T_3), 0.75% KPA (T_4), 1.00% KPA (T_5), 1.25% KPA (T_6) and 1.50% KPA (T_7) respectively and the basal (T_1) was prepared without any addition of KPA. The source of the KPA was the

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M/s Aquagri Processing Pvt. Ltd., New Delhi, India. The basal and experimental diets contained 21.51% crude protein, 2950 kcal/kg metabolizable energy, 0.98% calcium, 0.43 % available phosphorus, 1.21% lysine, 0.48% methionine and 0.96% threonine respectively.

Table 1 - Ingredient of starter diet (0-21d).

Ingredients (kg/100kg)	Starter diet (0-21d)						
	T_1	T_2	T_3	T_4	T_5	T_6	T_7
Maize, yellow	51.48	51.23	50.99	50.74	50.49	50.23	49.99
KPA[$]	0	0.25	0.5	0.75	1	1.25	1.5
Soyabean meal	40.3	40.3	40.3	40.3	40.3	40.3	40.3
Rapeseed meal	3	3	3	3	3	3	3
Oil	2	2	2	2	2	2	2
Limestone powder	0.9	0.9	0.9	0.9	0.9	0.9	0.9
DCP[1]	1.6	1.6	1.6	1.6	1.6	1.6	1.6
Salt	0.3	0.3	0.3	0.3	0.3	0.3	0.3
DL-Meth	0.1	0.1	0.1	0.1	0.1	0.1	0.1
L-lysine HCl	0.1	0.1	0.1	0.1	0.1	0.1	0.1
TM. Premix1*	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Vit Premix2**	0.015	0.015	0.015	0.015	0.015	0.015	0.015
B complex premix#	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Ch. Chloride, 60%	0.05	0.05	0.05	0.05	0.05	0.05	0.05

Table 2 - Ingredient of finisher diet (22-42d).

Ingredients (kg/100kg)	Finisher diet (22-42d)						
	T_1	T_2	T_3	T_4	T_5	T_6	T_7
Maize, yellow	57.09	56.84	56.59	56.34	56.09	55.84	55.59
KPA[$]	0	0.25	0.5	0.75	1	1.25	1.5
Soyabean meal	34.2	34.2	34.2	34.2	34.2	34.2	34.2
Rapeseed meal	3	3	3	3	3	3	3
Oil	2.7	2.7	2.7	2.7	2.7	2.7	2.7
Limestone powder	0.8	0.8	0.8	0.8	0.8	0.8	0.8
DCP[1]	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Salt	0.3	0.3	0.3	0.3	0.3	0.3	0.3
DL-Meth	0.17	0.17	0.17	0.17	0.17	0.17	0.1
L-lysine HCl	0.1	0.1	0.1	0.1	0.1	0.1	0.1
TM. Premix1*	0.1	0.1	0.1	0.1	0.1	0.1	0.15
Vit Premix2**	0.15	0.15	0.15	0.15	0.15	0.15	0.015
B complex premix#	0.015	0.015	0.015	0.015	0.015	0.015	0.05
Ch. Chloride, 60%	0.05	0.05	0.05	0.05	0.05	0.05	0.05

Trace mineral premix supplied Mg- 300, Mn- 55, I- 0.4, Fe- 56, Zn- 30 and Cu- 4 mg kg^{-1} diet.
**Vitamin premix supplied vitamin A, 8250 IU; vitamin D_3, 1200 ICU; vitamin K, 1 mg; vitamin E, 40 IU;
B-complex premix supplied vitamin B_1, 2 mg; vitamin B_2, 4 mg; vitamin B_{12}, 10 mcg; niacin, 60 mg; pantothenic acid, 10 mg; choline, 500 mg kg^{-1} diet; [$]KPA= *Kappaphycus alvarezii*; DCP[1]= Di-calcium phosphate

Body weight changes were recorded every three weeks (0-3 and 4-6 wks) during the experimental period. A weighed quantity of the respective diet was offered ad-lib daily in the morning and the residue was weighed next day to determine pen feed intake. Weekly and period wise feed conversion ratio (FCR) of birds was determined.

At 28 d of age, 8 birds/dietary treatment (56 birds in all) were inoculated intravenously with 1.0 ml of 1% sheep red blood cells (SRBC) suspension to investigate the effect on the humoral immune response and, at 35 d of age, 0.2 ml PHA-P mitogen (1 mg/ml PBS) was injected intra-dermally into the left foot web (another 8 birds/dietary treatment) for measurement of the cell-mediated immune response. Blood samples from 10 birds/treatment (n=70) were randomly collected at 42 d into sterile glass tubes without anticoagulant. Serum

was separated by centrifugation at 1512 g for 10 minutes and decanted into plastic vials, and then stored at -20^{0}C for estimation of serum enzymes alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), kidney function test (creatinine and uric acid), total cholesterol, total protein and albumin using commercially available biochemical kits (Span Diagnostics, India).

Data were tested for significance by SPSS- 20 in a completely randomized design, and means were compared using Duncan's (Duncan, 1955) multiple range test at $P < 0.05$.

III. RESULTS

Body weight gain (g) was significantly ($P<0.05$) improved in growing (21-42 d) and overall (0-42 d) phases in T_7 compared to the control and other treated groups; however feed intake (g) and feed conversion ratio (FCR) did not differ significantly ($P>0.05$).

Table 3 - Effects of dietary inclusion of *Kappaphycus alvarezii* on production performance of broiler chickens.

Group	Body weight gain (g)			Feed Intake (g)			Feed conversion ratio		
	0-21d	21-42d	0-42d	0-21d	21-42d	0-42d	0-21d	21-42d	0-42d
T_1	492.7	915.6^a	1408.3^a	845.6	1884.1	2575.8	1.71	2.05	1.83
T_2	495.8	937.8^a	1433.6^a	862.8	1896.1	2609.1	1.74	2.02	1.82
T_3	505.5	936.2^a	1442.2^a	879.6	1900.4	2509.5	1.74	2.03	1.74
T_4	490.9	960.1^{ab}	1450.9^a	829.6	1955.4	2539.0	1.69	2.04	1.75
T_5	484.9	964.7^{ab}	1449.6^a	806.4	1978.6	2568.9	1.66	2.05	1.77
T_6	533.9	1026.2^b	1560.1^b	865.0	2124.5	2699.0	1.62	2.07	1.73
T_7	513.9	1021.1^b	1535.1^b	827.5	2052.5	2640.4	1.61	2.01	1.72
SEM	6.20	11.5	19.56	5.9	23.12	28.45	0.001	0.002	0.001
P-value	0.319	0.038	0.027	0.057	0.076	0.240	0.052	0.062	0.057

Mean values bearing the same superscript in a row did not differ significantly ($P< 0.05$).
T_1 = Basal diet; T_2 = 0.25 % KPA; T_3= 0.50 % KPA; T_4= 0.75 % KPA; T_5= 1.00 % KPA; T_6= 1.25 % KPA; T_7= 1.50 % KPA

The haem agglutination (HA) titre to sheep red bold cells (SRBC), an index of humoral immunity, and the mitogenic response to PHA-P measured as the foot pad index (FPI), an index of cell-mediated immunity were significantly improved ($P<0.05$) by dietary supplementation of 1.5% KPA when compared to other treated groups.

Table 4 - Effects of dietary inclusion of *Kappaphycus alvarezii* on immune response and blood biochemical parameters of broiler chickens.

Group	Immune response		Blood biochemical parameters							
	CMI$^{\#}$ (mm)	$HA titre (log2)	Protein (g/dl)	Albumin (g/dl)	ALP* (IU/L)	ALT** (IU/L)	AST*** (IU/L)	Cholesterol (mg/dl)	Uric acid (mg/dl)	Creatinine (mg/dl)
T_1	0.50^a	1.92^a	3.79	1.39^a	205.15	52.23^b	26.73^a	161.36^b	1.55^b	0.24
T_2	0.51^a	1.94^a	4.34	1.42^a	210.86	52.14^b	27.04^a	160.90^b	1.52^b	0.25
T_3	0.54^{ab}	1.98^a	4.26	1.44^a	207.17	47.25^{ab}	28.88^a	156.74^{ab}	1.50^b	0.27
T_4	0.54^{ab}	2.01^a	4.49	1.48^{ab}	216.65	48.43^{ab}	28.96^{ab}	154.50^{ab}	1.44^{ab}	0.24
T_5	0.55^{ab}	2.07^{ab}	4.43	1.46^{ab}	214.03	47.22^{ab}	31.74^{ab}	155.12^{ab}	1.40^{ab}	0.23
T_6	0.54^{ab}	2.09^{ab}	4.81	1.49^{ab}	183.38	44.49^a	32.20^{ab}	152.63^{ab}	1.42^{ab}	0.23
T_7	0.58^b	2.26^b	4.56	1.57^b	186.27	44.17^a	33.48^b	149.32^a	1.28^a	0.22
SEM	0.05	0.07	2.17	0.16	5.14	0.17	0.30	5.12	0.10	0.09
P-value	0.021	0.032	0.064	0.027	0.076	0.024	0.039	0.032	0.017	0.119

Mean values bearing the same superscript in a row did not differ significantly ($P< 0.05$).
T_1 = Basal diet; T_2 = 0.25 % KPA; T_3= 0.50 % KPA; T_4= 0.75 % KPA; T_5= 1.00 % KPA; T_6= 1.25 % KPA; T_7= 1.50 % KPA*
ALP= Alkaline phosphatase; **ALT= Alanine aminotransferase; ***AST= Aspartate aminotransferase; CMI= Cell mediated immunity; $HA=Haem-agglutination.

No significant (P>0.05) differences were observed in total protein, creatinine and alkaline phosphatase (ALP) concentration; however, significant (P<0.05) increases in albumin and aspartate aminotransferase (AST) concentration, and decreased (P<0.05) alanine amino transferase (ALT), cholesterol and uric acid concentrations in T_7 (1.5% KPA) group compared with control birds and other KPA treated groups were observed.

IV. DISCUSSION

There are very few reports available in the literature regarding the effect of KPA in poultry diets. In the present study, KPA had a positive effect on growth performance, which was associated with improved immunity. Microalgal KPA had excellent antioxidant properties in the linoleic acid system, and also, ferrous ion-chelating activity which may influence production performance and immune response of broilers (Abirami and Kowsalya, 2011).
It is not clear how KPA enhances immune responses; one possibility is through greater antioxidant property which may protect the membranes and organelles of the lymphocytes from the detrimental effects of pro-oxidants. Moreover, KPA has some beneficial effect on immune characteristics i.e., lymphocytes, IgA, IgM and IgG concentrations which may be involved in modifying the metabolism of arachidonic acid to prostaglandin precursors or related compounds, enhancing immune responses by reducing the endogenous production of prostaglandin (Kang et al. 2013). KPA is rich in polysaccharides, minerals, proteins and vitamins. The inclusion of 1.5% KPA in broiler chicken diets was associated with significant decreases in blood concentration values of cholesterol, uric acids and alanine amino transferase (ALT). The decreased blood cholesterol seen in the present study was presumably due to the presence of more fibre, which may have inhibited hepatic cholesterol synthesis from fermentation metabolites by intestinal microflora. Fibre fermentation produces volatile fatty acids, including propionic acid, which is immediately absorbed through the hepatic portal vein and is transported to the liver where it inhibits the activity of HMG-CoA-reductase and, in turn, the rate of cholesterol biosynthesis (Stipanuk, 2012). In the present study, albumin level increased significantly after supplementation with 1.5% KPA suggesting that KPA had hepato-protective effects which may be exploited as growth promoters in the broiler chicken; positive outcome on the performance, irrespective of the type and level of seaweed used, might be due to a beneficial antimicrobial effect apart from positive impact on structural health of the small intestines, thereby facilitating nutrient absorption and growth performance in broiler chickens.

Thus it is concluded from this study that *Kappaphycus alvarezii* (KPA) @ 1.5% inclusion in the diet improved performance, immunity and blood biochemical parameters of broiler chickens.

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CHELATED COPPER COMPARED TO ANTIBIOTICS EFFECT ON GUT HEALTH IN BROILERS

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Summary

In this study the specific interventions of chelated copper methionine hydroxy analogue (cuMHAC) and a combination of cuMHAC and an effective foregut acidifier were tested on broiler chickens. The treatment effects were studied against birds fed diets containing antibiotics or no treatment. The negative control containing no antibiotics performed as well as the antibiotic and cuMHAC, with no significant treatment effect found in any performance indicators including growth, feed intake, carcase weight or feed conversion efficiency. The treatments including cuMHAC alone and cuMHAC with acid water resulted in less feed required to reach equivalent bodyweight to other treatments in the first 10 days of growth. Performance by day 35 was equal across all treatments. Significant differences in intestinal architecture were found between negative control which had least villus to crypt ratio $P < 0.01$ compared to all other treatments. Foot pad lesions were least evident in the antibiotic treatment and in both cuMHAC treatments. No treatments had significant deviation from antibiotic control in flock uniformity, antibody titer response, intestinal bacterial enteritis score, carcase weight and dressing percentage, salmonella counts in litter or litter score in this study.

I. INTRODUCTION

The following statement was made by Landers et al., (2012) in a public health report. “Although the majority of antibiotic use occurs in agricultural settings, relatively little attention has been paid to how antibiotic use in farm animals contributes to the overall problem of antibiotic resistance”. Some classes of antibiotics are becoming less effective due to overuse and microbial adaptation leading to resistance to treatment. The pool of available, effective antibiotics is shrinking as this resistance increases. The European Union, Thailand and Indonesia are some of the largest food producing jurisdictions that have banned or restricted the prophylactic use of antibiotics as a growth promoter in all animal production. There is already restricted use in Mexico, Japan, South Korea and Vietnam with more countries planning phase out of antibiotics with China pledging to ban AGPs in the next 5 years. Routine addition of antibiotics in broiler feed to reduce disease incidence and improve efficient growth is now banned in many jurisdictions globally. As producers look to a reliable antibiotic alternative, it is clear, as shown by Dibner and Richards (2005), that no single intervention will support the immune system and growth of broilers independently. Alternatives to antibiotics are being used and sought to ensure the health and performance of broilers, layers and other farmed species across the world. There are many feed ingredient alternatives that have been tested and shown variable responses. Mehdi et al. (2018) cited the following “among these, the most popular are probiotics, prebiotics, enzymes, organic acids, immunostimulants, bacteriocins, bacteriophages, phytogenic feed additives, phytoncides, nanoparticles and essential oils”. Second only to the trace mineral zinc in enzyme regulation, copper has been shown to have a positive effect on broiler health. Yazdankah et al. (2014), in a paper concerned with microbial co-resistance, noted super-dosing of copper in inorganic forms such as copper sulphate is used routinely in both swine and broiler operations. In addition to the importance

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in cellular respiration, antioxidant function and iron transport, copper supports the immune system where neutrophils rely upon the 'respiratory burst' to generate free radicals such as superoxide (O2 −), which kill engulfed pathogens (Suttle, 2010).

II. METHODS

A total of 672 male Arbor Acres Plus broilers were randomly assigned to one of 4 treatments (Table 1). This consisted of 168 birds in each treatment in 8 pens containing 21 birds each. These birds were fed for 35 days and then processed. Birds were housed on re-used litter. Diets were based on corn and soy bean meal containing maximum 7% corn DDGS with 3.4% meat and bone meal. All diets were identical in makeup except the cuMHAC treatments which were balanced for methionine content and contained no supplemental inorganic copper.

Treatment one contained the antibiotic zinc bacitracin as positive control. Treatment two contained no antibiotic or other additive. Treatments three and four included copper in the form of cuMHAC at 30ppm. In treatment four, water acidification was achieved using a blend of liquid methionine hydroxy analogue, formic and propionic acid at 0.2 ml per litre to achieve a drinking water pH of four. Measurements taken at the beginning of the study were litter score, flock uniformity, diet proximate analysis and litter score for *Salmonella* levels. Microbiota analysis was done by sacrificing 1 bird per pen at 21 days and collecting ileum contents. These samples were tested for *Lactobacillus* and *Clostridium* content. Histology samples were also fixed at this time. Antibody titration against Newcastle disease (ND) using hemagglutination inhibition (HI test), infectious bronchitis (IB) (HI test) and infectious bursal disease (IDB) (ELISA) was measured in 2 birds per pen at 28 days. Intestinal health was scored by an independent veterinarian at 35 days on 2 birds per pen at processing. Carcase measurements at processing included carcase yield, foot pad lesions, tibial head lesions and liver weight. Statistical analysis was conducted using Duncan's multiple range test where $P < 0.05$ was considered to be significant.

Table 1 - Treatments.

Treatment	Dietary inclusion
1	Antibiotic control, contains zinc bacitracin positive control
2	Negative control contains no antibiotics or additive treatment negative control
3	Contains 30ppm copper as methionine hydroxy analogue chelate
4	Contains 30ppm copper as methionine hydroxy analogue chelate, birds received water acidifier target pH of 4

III. RESULTS

As seen in Table 2, although there was a numerical advantage for the cuMHAC treated groups in FCR at 10 days, no treatment resulted in significant improvement to bodyweight, feed conversion or uniformity by day 35 of the study.

Table 2 - Feed conversion efficiency.

	FCR 0-10d	FCR 0-35d
T1	1.016	1.348
T2	1.017	1.354
T3	1.007*	1.342
T4	1.007*	1.347

* numerically different not significantly different (P = 0.14) from other treatments

Results at 21 days when small intestine contents were analysed for *Lactobacillus spp* and *Clostridium perfringens* content showed numerical differences across treatments with

cuMHAC treatments trending higher for both colonies but no statistically significant differences and with no differences seen between antibiotic and negative control groups. This suggested little to no challenge to intestinal health; this was reinforced with no difference seen in bacterial enteritis score, antibody titre response or litter *Salmonella* count between treatments. Thofner et al. (2019) discuss the use of foot pad lesions as a measure of welfare. Both foot-pad lesions and tibial head lesions are typical health concerns in modern broiler production leading to lameness, ethical destruction or a reduction in healthy pieces including saleable paws (feet) at processing. Interesting trends emerged in this study among the treatment groups with foot pad lesions least evident in antibiotic treatment and in both cuMHAC treatments while negative control had numerically ($P < 0.1$) fewer healthy (score zero) lesions. This trend continued for tibial head lesions where incidence tended to be greater in the negative control group (39% higher incidence $P < 0.8$) than in the antibiotic control. All carcase pieces and organ weights were similar.

Table 3 - Results at harvest.

	Dressing %	Liver weight	Footpad clean, % score 0	Tibia head, % affected
T1	76.27	2.87	58.3	39.6
T2	76.15	2.85	50.0*	64.6**
T3	76.20	2.78	60.4	39.6
T4	75.61	2.91	56.3	31.3

Footpad clean, score 0 - shows no sign of injury or dermatological damage; *P < 0.1; **P < 0.08.

Significant differences in intestinal architecture were found between negative control which had least villus to crypt ratio ($P < 0.01$) compared to all other treatments as seen in Table 4.

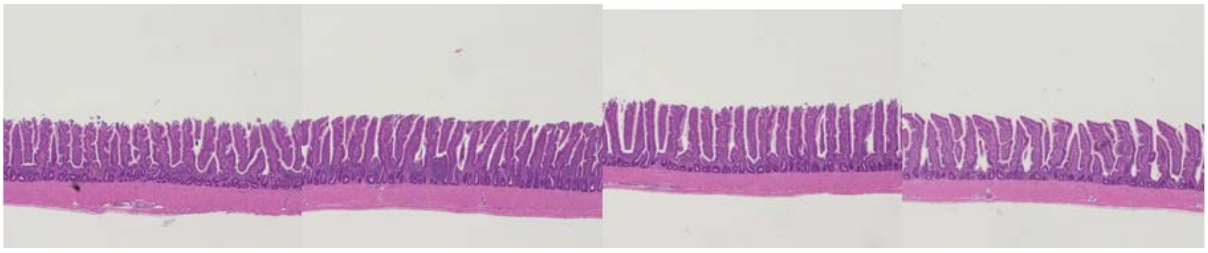

Figure 1 - Negative control

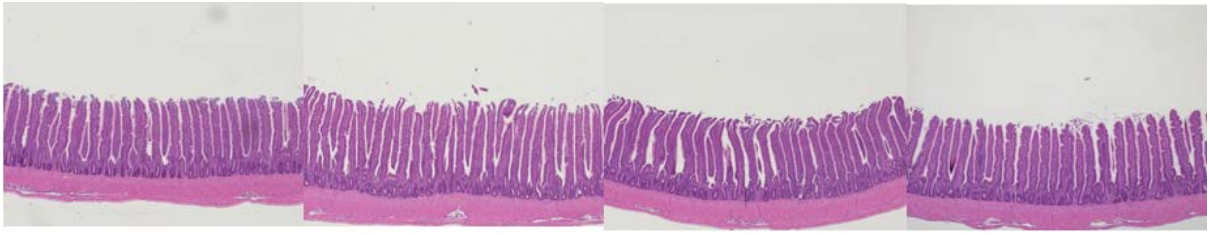

Figure 2 - Histology showing greater development of villus and greater villus height to crypt depth ratio

Table 4 - Intestinal architecture.

	Villus height	Villus height to crypt depth ratio
T1	566	4.54
T2	515	4.03*
T3	561	4.59
T4	562	4.63

* significantly different from other treatments P < 0.01.

IV. DISCUSSION

Although the study was conducted in an environment which had been designed to allow potential for antimicrobial activity due to a moderate degree of performance pressure, the birds remained healthy. The birds were housed on re-used litter, offered feed with maximum levels of DDGS and MBM and were held at maximum pen density. This study did give an excellent indication of how the cuMHAC fed birds responded when very little performance pressure was applied. The key indicator of the healthy flock was the equivalent performance of the negative control treatment T2 to the zinc bacitracin fed group T1. The only area of difference between the antibiotic treatment and control was in the tibial head lesion score ($P < 0.08$) which might suggest some level of gut barrier failure. Results from this study suggest that the most consistent performance improvement can be gained by including copper hydroxy analogue or copper hydroxy analogue with an effective foregut acidifier. Arbe and Bekker (2017) showed how inclusion of copper methionine hydroxy analogue could increase the performance of broiler birds at lower inclusion levels than inorganic salts and Hassan et al (2010) showed the benefit of organic acid inclusion without an antibiotic growth promoter which supports the trends seen in this study. The other notable trend was for birds that grew more efficiently during the critical first 10 days to have greater carcase integrity at 35 days, resulting in healthier foot pads and tibia joints with fewer lesions.

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DIETARY MICROBIAL MURAMIDASE IMPROVES GROWTH PERFORMANCE ALONE OR IN COMBINATION WITH ANTIBIOTIC GROWTH PROMOTERS

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The gastro-intestinal tract (GIT) of poultry has a complex and dynamic microbial community consisting primarily of bacteria whose cell wall contains the structural polymer peptidoglycans (PGNs). Bacterial cell wall recycling is a process whereby bacteria degrade their own wall during growth in order to recover released constituents by active transport. These nutrients are then reutilized to either rebuild the wall or to gain energy (Mayer, 2012). However, in both normal and a challenged GI tract environment, PGNs can be accumulated. The consequence of PGNs accumulation on the intestinal lumen remains to be investigated, but it could be speculated that accumulation of bacterial cell wall fragments in the gut could impair nutrient digestion and absorption, and negatively affect animal performance. Muramidases are natural enzymes that hydrolyze PGNs from the bacterial cell wall. Recent studies (Lichtenberg *et al*. 2017; Goodarzi Boroojeni *et al*. 2019; Sais *et al*. 2019) have reported positive effects on broiler growth performance by supplementation with a novel microbial muramidase (MUR) which could hydrolyze the PGNs from bacterial cell wall fragments. The goal of the experiment was to evaluate the effect of a microbial MUR with and without antibiotic growth promoters (AGP), on broiler growth performance.

A 42-day feeding trial was conducted with a male flock of 1600 Cobb 400Y chicks randomly allocated to 64 floor pens (25 birds each). Broilers were fed corn-soybean-meat and bone meal-based diets containing Robenidine as coccidiostat. A three-phase dietary program and four experimental pelleted diets were used: a negative control diet (NC), a NC diet + Bacitracin methylene disalicylate (BMD, 50 ppm), NC diet + MUR (25000 LSU(F)/kg) and a NC diet + BMD (50 ppm) + MUR (25000 LSU(F)/kg). Growth performance parameters were recorded at day 0, 14, 28 and 42 of the study. Data were analyzed by two-way ANOVA and means were compared by the Student-Newman-Keuls.

In the present study, the inclusion of MUR in the diet improved body weight gain ($P < 0.001$) but not when an AGP was included in the diet ($P = 0.923$), and no interaction was found between AGP and MUR ($P = 0.364$). MUR supplementation significantly ($P < 0.001$) increased BWG by 2.4% and 3.2% compared to the NC and NC + BMD, respectively, but no differences were observed when NC + BMD + MUR. Regarding FCRc, the inclusion of MUR showed again a significant improvement ($P < 0.001$) but not when AGP was included in the diet ($P = 0.472$) but this time an interaction between AGP and MUR was observed ($P = 0.014$) indicating that AGP only improves FCR when it is included with MUR. This combination showed the best FCRc value and one explanation could be that AGPs promote bacterial turnover thereby increasing the PGNs in the gut and consequently providing more substrate for the microbial muramidase.

In conclusion, dietary microbial muramidase alone or in combination with AGPs improves broilers performance.

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BACILLUS SUBTILIS SUPPLEMENTED DIET IMPROVES WEIGHT GAIN AND CAECAL LUMINAL MICROBIOTA IN MEAT CHICKENS

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Necrotic Enteritis (NE) outbreaks cause an estimated US$6 billion financial loss *per annum* to the global poultry industry (Wade & Keyburn, 2015). With discontinuation of antibiotic growth promoters (AGPs) in Europe and implicit withdrawal of AGPs worldwide, a global research effort has been made to find alternative solutions to minimise diseases in animal production including NE. The current study investigated the benefits of a *Bacillus subtilis* derived probiotic (Bs29784) in NE challenged broiler chickens. The study used 312 male day-old Ross 308 in floor pens in separated blocks of the same room from d 0 to d 35. The treatments groups included: two control diets without or with NE challenge (CtrlNC and CtrlNE, respectively); two treatment diets of probiotic and antibiotic supplements both with NE challenge (ProNE and AntNE). Diets comprised standard wheat and soybean-meal based control diet for CtrlNC and CtrlNE, and addition of respective additives for either ProNE (Bs29784 500 mg/kg) diet or AntNE (Zn Bacitracin 4.4 mg/kg) diet. NE challenge procedures commenced with *Eimeria* spp. 1 mL/bird *per os* at d 9 with a suspension of 5000 sporulated oocysts of E. *acervulina*, and *E.maxima*, and 2500 sporulated oocycts of *E. brunetti* and *Clostridium perfringens* EHE-NE18 (approx. 10^8 CFU/mL) and 1 ml/bird *per os* at d 14 and d 15 (Wu et al., 2010). Performance was measured, and samples for 16S next-generation sequencing (NGS) in caecal content and gene expression analysis from jejunal tissue were collected on d 16 and/or d 35. Data analysis applied a one-way ANOVA and significance at $P < 0.05$ with Tukey`s test.

NE challenge (CtrlNE) significantly suppressed the performance parameters (27% weight gain reduction, 13 points FCR increase at d 16, and 12% weight gain reduction, 6 points FCR increase at d 35) compared to the CtrlNC. By d 35, the probiotic and antibiotic treatments enabled significantly higher weight gain than the CtrlNE. Caecal microbiota d 16 showed reduced OTUs counts in all the challenged groups regardless of additives, but no difference was observed at d 35. For microbial abundance analysis, Venn diagram showed that each group has their specific OTUs at d 16 (6, 3, 3 and 2 for CtrlNC, AntNE, ProNE and CtrlNE, respectively), whereas only CtrlNC showed 1 specific OTU at d 35. Principal component analysis (PCA) of OTUs abundance revealed different diversity between the respective treatment groups. The ProNE and AntNE grouped together while the CtrlNC and CtrlNE grouped separately at d 16, indicating the similarity of OTUs between the ProNE and AntNE birds that were however different from both CtrlNC and CtrlNE birds. Interestingly, the OTUs structure at d 35 changed with distinct grouping of the CtrlNC or AntNE from CtrlNE and ProNE, latter two grouped closely although minimal overlapping. This may imply recovered microbiota balance in both the CtrlNE and ProNE while the strong antimicrobial effects in AntNE induced the different microbiota structure. The taxonomic composition indicated an increase of *E. coli* in all challenged groups at d 16, but a balanced composition was observed among all groups at d 35 with abundance of *F. prausnitzii* in ProNE. Besides, ProNE treatment exhibited upregulation of the genes at d 16 ($P < 0.05$): *CLDN1, IL12-b, MUC5ac, TGF-4B, TLR21, TLR5, JAM2 and INFy* genes, suggesting enhanced immunity and intestinal integrity. It is suggested that Bs29784 may enable improved health of meat chickens under NE challenge.

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USE OF A PHYTOGENIC BLEND OF CINNAMALDEHYDE AND THYMOL ALONE OR IN COMBINATION WITH A BACILLUS PROBIOTIC IMPROVES PERFORMANCE OF BROILERS DURING HIGH CHALLENGE SITUATION

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Summary

The current study looked at the effects of including a phytogenic blend (EO), alone or in combination with a *Bacillus* based probiotic (EOPRO) or an antibiotic growth promoter (AGP) in broilers. The unchallenged birds fed the enzyme treatment alone performed as expected. The challenge administered in this study was used to induce severe dysbacteriosis; birds were inoculated with coccivac (day 7), *E. coli* (days 14 and 21) and *C. perfringens* (days 13 and 15). The challenge resulted in significant negative effects on performance and the Broiler Index (calculated using bodyweight, feed conversion ratio, FCR and mortality data) versus the unchallenged birds. The FCR improvements demonstrated with the EO and EOPRO treatments indicate that these challenged birds could partially recover from their given challenge. The Broiler Index was maintained at the level of the unchallenged birds in challenged birds fed EO or EOPRO. In contrast, no performance improvements were seen with the AGP. Birds fed the EO and EOPRO supplements had improved performance and were able to cope with the administered challenge more successfully due to a healthier gut at the time the challenge was administered.

I. INTRODUCTION

Phytogenics and probiotics are gaining more attention in the animal industries due to market trends to reduce antibiotics use, whilst still preventing disease outbreaks and maintaining, or even improving, animal performance. Phytogenics have been shown to inhibit non-beneficial, potentially pathogenic bacteria such as *E. coli* (Ouwehand et al., 2010, Bento et al., 2013), positively influencing the gut microbiome and inhibiting *C. perfringens* populations. *Bacillus* based probiotics have been shown to influence gastro-intestinal tract (GIT) microbial populations and reduce Avian Pathogenic *E. coli* counts in the GIT of broilers (Wealleans et al., 2017). The modes of action of cinnamaldehyde and thymol in terms of influencing gut health differ from *Bacillus* probiotics. The phytogenics have direct antibacterial effects (Bento et al., 2013) whereas *Bacillus* have a number of different modes of action which include outcompeting non-beneficial bacteria, encouraging growth of beneficial bacteria and aiding development of the immune system. Therefore, it is expected that their modes of action may be complementary and that using the phytogenic and probiotic together may lead to improved performance of broiler chicks during challenge situations. Few studies to date have looked at combining probiotic and phytogenic treatments.

II. MATERIAL AND METHODS

A total of 2160 day-old Lohmann Indian River chicks were randomly allocated to 5 dietary treatments with 12 replicate pens per treatment (36 birds/pen). Birds were fed over 2 dietary phases: starter (1 to 21 days) and grower/finisher (22 to 35 days). All diets were formulated to meet the birds' nutrient requirements and were fed *ad libitum* as crumble (starters) or pellet

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(finishers). All diets contained *Buttiauxella* phytase (Axtra® PHY, DuPont Animal Nutrition) at 1000 FTU/kg of feed and a combination of xylanase, amylase and protease (Avizyme® 1505X, DuPont Animal Nutrition) at 200 g/kg of feed (to provide 1840 U xylanase/kg, 320 U amylase/kg and 3200 U protease/kg of feed). The control diets (Table 1) were reduced by 0.187% phosphorus, 0.199% calcium, 126 kcal ME, 0.028% methionine plus cysteine and 0.045% lysine in line with the enzyme manufacturers recommendations for reformulation.
One group of birds was fed the control diet and was not disease challenged (UC). Challenged birds (CC) were inoculated with Coccivac, *C. Perfringens* and *E. coli* according to the schedule in Table 1. The challenged birds were also fed the control diet, or this diet supplemented with either Antibiotic Growth Promotor (AGP) at 50g/tonne, phytogenic blend (EO) at 100 g/t feed (to provide 4.5 g of cinnamaldehyde and 13.5 g of thymol/tonne of feed), or a combination (EOPRO) of EO at 100 g/t feed and 3 strains of *Bacillus* probiotic at 60 g/t feed (to provide 150 000 CFU/g feed). Bodyweight and feed intake were measured at the start and end of each phase. FCR and Broiler Index (BI) were then calculated. BI was calculated as:

[ABW / Days of Age * (100 - (%) DEPL) / FCR / 10] ;

in which: ABW = Average Body Weight; DEPL = Depletion.

Table 1 - Diet composition (g/kg).

Ingredient	Starter (1-21 days)	Grower/finisher (22-35 days)
Corn	311	402
Wheat	250	250
Soybean meal	235	147
Corn DDGs	60	0
Wheat Bran	35	62
Poultry by-product meal	30	30
Feather meal	30	50
Palm Oil	21	35
Limestone	10.6	8.8
MCP	1.17	0.26
Salt	1.24	1.15
L-Lysine HCl	4.55	4.81
DL-Methionine	3.18	2.39
L-threonine	1.20	1.07
L-valine	0.38	0.08
Sodium bicarbonate	2	1.75
Mineral mix	0.65	0.60
Choline Chloride	0.26	0.46
Vitamin Mix	2.2	2.2
Axtra® PHY*	0.1	0.1
Avizyme 1505X**	0.2	0.2
Calculate analysis (g/kg)		
Crude Fibre	29.6	26.5
Crude Protein	238	207
ME (MJ/kg)	12.0	12.7
Digestible crude protein	203.9	175.2
Digestible lysine	12.7	10.7
Digestible methionine	6.0	4.7
Digestible tryptophan	2.2	1.8
Digestible threonine	8.2	7.1
Digestible valine	10.1	8.8

*To supply 1000 FTU/kg
** To supply 1840 U xylanase, 320 U/kg amylase and 3200 U/kg protease

Gut samples were taken on day 21 and day 35 from 24 birds/replicate (2 birds per pen; one male, one female). Lesions in three locations were scored: duodenum (*E. acervulina*), ileum (*E. maxima*) and caeca (*E. tenella*) from 0-4; good-bad (Johnson and Reid, 1970). Also, Bacterial Enteritis Scores (BES) were scored by evaluating different parameters either 0 (good) or 1 (bad) and then added together per pen; a lower score is indicative of better gut health

Table 2 - Challenge protocol.

Day of challenge	Challenge
7	Coccivac 10 at 10x dose
13	10^6 CFU *Clostridium perfringens*
14	10^9 CFU *Escherichia coli*
15	10^6 CFU *Clostridium perfringens*
21	10^9 CFU *Escherichia coli*

III. RESULTS

The challenge applied during the trial was effective and negatively impacted performance, reducing bodyweight gain by 4.8% and FCR by 6 points (3.8%, $P < 0.05$). The challenge also resulted in a 3.2% increase in mortality and a significant decrease in the Broiler Index by 11.3% ($P < 0.05$).

Table 3 - Effects of phytogenic and probiotic supplementation on broiler growth performance from 1-35 days.

Treatment	Final weight (g)	Average Daily Gain (ADG, g/b/d)	Feed Intake (g/b/d)	FCR	Broiler index	Mortality (%)
UC	2293a	64.2a	101.8	1.59a	388a	4.1
CC	2188ab	61.1ab	100.7	1.65b	344b	7.3
CC + AGP	2162b	60.4b	98.9	1.64b	345b	6.6
CC + EO	2215ab	61.9ab	100.4	1.62ab	359ab	6.1
CC + EOPRO	2238ab	62.6ab	101.2	1.62ab	362ab	6.3
P value	0.022	0.023	NS	0.001	0.001	NS

abc Means within columns not sharing common suffixes are significantly different at the 5% level of probability
NS = not significantly different

Challenged birds fed the EO and EOPRO treatments presented numerically improved performance versus the challenged control birds. Average daily gain was improved with the EO and EOPRO treatment compared to the challenged birds fed the control treatment, by 1.31% and 2.45%, respectively. Final bodyweight tended to improve by 1.2% and 2.2% with the EO and EOPRO treatment versus the challenged control and was not significantly different from the unchallenged birds. FCR was also numerically improved by 3 points in birds that were given the disease challenge with both the EO and the EOPRO treatments and for both the EO and EOPRO treatments ADG and FCR were not significantly different to the unchallenged control birds. In contrast, the AGP treatment did not result in any performance improvements in the challenged birds, with both the EO and EOPRO treatments numerically improving performance (ADG, FCR and broiler index) versus the AGP.

Broiler Index tended to improve (by 4.36% and 5.23%, respectively) for the EO and EOPRO treatments compared to the challenged control. Gut health scores did not differ among treatments.

Table 4 - Effects of phytogenic and probiotic supplementation on broiler gut health scores (day 35).

Treatment	Duodenal Lesion scores (*E.acervulina)*	Ileal Lesion scores (*E.maxima)*	Caecal lesion scores (*E.tenella)*	Bacterial enteritis scores (BES)
UC	0.000	0.635	0.500	4.417
CC	0.000	0.542	0.417	4.417
CC + AGP	0.042	0.750	0.292	5.083
CC + EO	0.042	0.375	0.500	4.167
CC + EOPRO	0.000	0.208	0.375	4.833

IV. DISCUSSION

The challenge administered in the current study was severe, combining an *Eimeria*, *E. coli* and *C. perfringens* disease model. The results of the study indicated that when birds were fed either the EO treatment or the combination of EOPRO, the performance impact of the challenge was reduced and growth performance was maintained at the same level as unchallenged broilers fed the same diet. The final body weight and ADG tended to be greatest when the phytogenics and probiotic were fed in combination. The EO and *Bacillus* used in the study have been shown to positively influence the microbiome of broilers (Ouwehand et al., 2010, Bento et al., 2013, Wealleans et al., 2017) by supporting beneficial microbial populations (e.g. lactic acid bacteria) and reducing potentially non-beneficial populations (e.g. *E. coli* and *C. perfringens*). There is some evidence the *Bacillus* strains can support the development of the avian immune system, making them better equipped to deal with intestinal challenges and studies have also indicated that a cinnamaldehyde and thymol combination can help modulate the immune system and improve intestinal immunocompetence in young broilers (Bento et al., 2013). Such modes of action could explain the positive performance effects noted in the current study, although these measurements were not taken directly. This is not the first time these additives have been documented to have a beneficial effect in challenge studies. Dersjant-Li et al. (2016) reported reductions in inflammatory responses, improvements in gut structure and a maintenance of bird performance to that of the UC in a coccidiosis/necrotic enteritis study. The current study provides supporting evidence for the potential to combine feed additive solutions in an antibiotic-free diet without negatively impacting performance during times of disease challenge. It is likely that the combined influences of the probiotics and phytogenics on the nutrition, microbiome and immune status of the animal will have contributed to a favourable health status and resulted in a more robust animal with the ability to deal with pathogenic challenges more quickly, to minimize their potential negative effects.

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ULTRASTRUCTURAL CHANGES IN THE ILEAL MUCOSA OF BROILERS EXPOSED TO NECROTIC ENTERITIS AND *BACILLUS AMYLOLIQUEFACIENS* H57

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The villi of the intestinal mucosa are lined by a single layer of columnar cells, comprising enterocytes, goblet and enteroendocrine cells, and various types of immune cells, each with a distinct function. Chicken gut mucosa is exposed to an enormous number of feed antigens, and pathogenic bacteria that often impair the intestinal barrier function. Ultrastructural examination of enterocytes, their organelles and other features, such as mitochondria, microvilli, and tight junctions sealing adjacent epithelial cells, can help to reveal the state of gut health. In chickens, disruption of the epithelium and disintegration of villi occur following gut infections, including necrotic enteritis (NE), which can cause severe damage. Birds with subclinical NE do not display clinical signs of disease, but can experience malabsorption and poor growth. Probiotics are being investigated as alternatives to antimicrobials, and research with the probiotic *Bacillus amyloliquefaciens* strain H57 (H57) has shown significant effects on performance and feed utilization. The aim of this study was to examine ultrastructural events occurring on ileal epithelium in broilers exposed to subclinical NE and fed H57 ($2x10^8$ spores/g feed). Subclinical NE was induced in chicks exposed to a high dose of *Eimeria* vaccine via drinking water, and *Clostridium perfringens* (CP) culture mixed with feed as described by Shini *et al*. (2020). On day 21, six birds per treatment were sampled and, ileal mucosa was evaluated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

The exposure of broilers to coinfection with *Eimeria* and CP caused subclinical NE as shown by lesions on the intestinal mucosa. Ultramicroscopic examination of ileal mucosa revealed significant villus damage, such as focal erosions of epithelial cells, exposure of lamina propria, and villous atrophy. The degree of damage was found to be higher ($P < 0.001$) in NE birds when compared to other treatments, and it was graded as 2.6 vs. 0.8 in NE vs. NE-H57 birds, respectively. There was a higher ($P < 0.05$) abundance of segmented filamentous bacteria attached to villi in birds exposed to NE. TEM revealed severe enterocyte damage, and loss of cellular integrity in NE birds. Mitochondria, in particular, were undergoing morphological alterations. In NE birds, mitochondria were irregular in form, containing electron-lucent regions of matrix, swollen or damaged cristae, most probably due to oxidative stress, resulting in increased ROS exposure and lowered ATP production. In control, H57, and NE-H57 birds, most mitochondria were round or elongated, with mild or no structural damage. Tight junctions were longer ($P < 0.001$) and more regular in the control and H57 treated birds when compared to NE birds. Tight junctions are crucial for the maintenance of epithelial barrier integrity. The results suggest that H57 contributes to the maintenance of intestinal barrier integrity and function. Establishing the links between the mucosa and probiotics will enable the development of new strategies to maintain gut health in the antibiotic-free era in commercial poultry.

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EFFICACY OF THE PROBIOTIC *BACILLUS AMYLOLIQUEFACIENS* H57 IN A CHICK SUBCLINICAL NECROTIC ENTERITIS MODEL

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Probiotics are showing promise as antibiotic replacements but more scientific evidence is required to validate their beneficial effects. Previous broiler feed trials conducted with *Bacillus amyloliquefaciens* strain H57 (H57) indicated variable bird performance (Bajagai, 2018). In these trials, birds were kept under "optimal" experimental conditions, where most of stressors were absent from the environment. There is some evidence that probiotics are best applied when a bird or animal is under stress, from infectious, nutritional or environmental stressors, and in commercial chickens, enteric infections are one of the most important environmental challenges in early life. Necrotic enteritis (NE), caused by *Clostridium perfringens* (CP), has become one of the most significant diseases in broilers. Subclinical NE, which is difficult to diagnose, can spread through flocks, resulting in substantial production losses. A subclinical NE model was developed in broilers to assess the efficacy of H57 in improving intestinal health and preventing NE. It was our hypothesis that, H57 spores administered in the diet during the first three weeks of life, will maintain intestinal health and improve feed utilisation.

Subclinical NE was induced in Ross broilers by exposing them to a high dose of *Eimeria spp.* vaccine (20x the manufacturer's recommended dose) via drinking water on day 9 post-hatch, and 5 days later to CP culture (strain EHE-NE18, CSIRO) mixed in the feed. In this factorial study there were six treatments (control, *Eimeria*-challenged, CP-challenged, NE-challenged, NE-H57, H57) each with six replicates of eight chicks. The control diet was supplemented with the probiotic ($2x10^8$ spores/g feed) throughout the study, and fed to two groups of birds, NE-H57 and H57. Data on performance, pasty vent, NE lesion scoring, and histopathology were used to assess effects of H57 on bird gut health.

There were no significant treatment effects on total body weight and feed intake. However, in NE-H57 birds, FCR was significantly improved when compared to NE birds (1.28 vs. 1.36; $P < 0.001$). In H57 birds, FCR was improved by 0.03 units when compared to controls (1.24 vs. 1.27; $P = 0.051$). NE birds had a higher occurrence of pasty vent, than *Eimeria*, CP or NE-H57 birds (41%, vs. 27%, 29%, 19%; $P < 0.001$). Lesion scores of NE birds were also higher ($P < 0.001$) than in challenged (*Eimeria*, CP, and NE-H57), and unchallenged birds (control and H57), 5.67 vs. 2.56, 2.78, 2.10, 1.17, 0.83, respectively). There was a strong correlation between pasty vent and lesion scores (Pearson's $r = 0.56$; $P < 0.001$). Microscopic evaluation of ileal mucosa showed mucosal damage and necrosis in NE birds. In contrast, villi from NE-H57 birds were normal in appearance, with no damage or infiltration with *Eimeria* or CP. It was concluded that H57 is more effective in birds subjected to an infectious challenge. NE-H57 birds had improved feed efficiency and maintained epithelial barrier integrity. This was confirmed with electron microscopy as described by Shini et al. (2020).

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EFFICACY OF SYNERGISTIC BLEND OF FEED ADDITIVES ON GROWTH PERFORMANCE, GUT HEALTH AND BIRD WELFARE IN BROILERS CHALLENGED WITH NECROTIC ENTERITIS

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A feeding study was conducted to examine the efficacy of a synergistic blend of feed additives on growth performance, livability, gut integrity, immunity, caecal microflora and footpad health in broilers challenged with subclinical necrotic enteritis (NE). Additives were: A) synergistic blend of medium chain fatty acids (MCFA), slow-release C12, target release butyrates, organic acids (OA) and a phenolic compound; B) synergistic blend of partly buffered OA with MCFA; C) synergistic blend of partly buffered OA with a high concentration of MCFA. A total of 1404 male Ross 308 chicks were assigned to 78 floor pens each stocked with 18 birds. A randomised complete block design was used with 6 treatments replicated 13 times and the treatments were: T1 - unchallenged group, without additives or in-feed antimicrobials; T2 - challenged group, without additives or in-feed antimicrobials; T3 - challenged group plus in-feed antimicrobial (Zinc bacitracin); T4 - challenged group plus additive A at 1.5, 1.5, 0.5 g/kg feed; T5 - challenged group plus additive B at 2.5, 2.0, 1.0 g/kg feed; T6 - challenged group plus additive C at 2.0, 1.5, 1.0 g/kg feed in starter, grower and finisher phases, respectively. Diets were based on wheat and soybean meal and were supplemented with xylanase and phytase. Challenged birds were given field strains of *Eimeria spp*. oocysts consisting of *E. acervulina* (5000), *E. maxima* (5000) and *E. brunetti* (2500) at d 9 and *Clostridium perfringens* (Cp) at d 14 (10^8 CFUs/mL). Mortality data were used to correct the FCR. Bird performance was measured from d 0 to 35. Serum fluorescein isothiocyanate dextran (FITC-d) was used as a leaky gut marker to measure gut integrity; immunoglobulins and caecal microflora were measured at d 16. Footpad health and litter quality (Kheravii et al., 2017) were scored at d 35.

The unchallenged group had higher feed intake (FI), body weight gain (BWG), lower FCR, and serum FITC-d concentration compared to NE challenged groups ($P < 0.05$). BWG and livability were not significantly different among the challenged groups. Birds supplemented with feed additives had lower FCR compared to T2 (overall, $P < 0.05$). On d 16, a higher concentration of serum FITC-d was observed in T2 compared to feed additives groups ($P < 0.05$). Birds challenged with NE had a higher level of serum IgA but no effects were observed in serum IgG and IgM levels. Birds fed additive C had lower counts of *Bacteroides spp*. compared to T2 ($P < 0.05$). Birds fed additives had lower counts of *Ruminococcus spp*. than T2 ($P < 0.05$). Cp counts were not significantly different between additives B, C, and in-feed antimicrobial groups ($P > 0.05$). Birds treated with feed additives had lower footpad dermatitis (FPD) and hock burn scores (HB) compared to T2 ($P < 0.05$). There was a tendency to improve the litter quality in the additive groups ($P = 0.072$). However, a strong positive correlation between litter quality and FPD ($r = 0.388$, $P < 0.0001$) and HB scores ($r = 0.581$, $P < 0.0001$) was observed. These findings suggest that additives A, B, and C were effective in alleviating the impact of NE as indicated by improved FCR, enhanced gut integrity and improved bird welfare. These results also demonstrated that the diet supplemented with additive C helped to maintain good gut health by altering the intestinal bacterial population.

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OVER-PROCESSED MEAT AND BONE MEAL DECREASED PERFORMANCE IN BROILERS CHALLENGED WITH SUBCLINICAL NECROTIC ENTERITIS

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A study was conducted to investigate the hypothesis that over-processing (OP) of meat and bone meal (MBM) exacerbates infection with necrotic enteritis in normal and high phytase diets. A total of 768 Ross 308 male chicks were randomly assigned to 8 treatments with 6 replicate floor pens each housing 16 chicks using a 2 × 2 × 2 factorial arrangement of treatments for 42 d. Factors were: NE challenge, no or yes; phytase* level, 500 or 5000 FTU/kg (both using 500 matrix values for Ca, P and Na of 1.6, 1.5 and 0.35 g/kg respectively); and MBM, Normal (as received) or OP (by autoclaving for 90 mins at 128^0C at 2 bars). Challenged birds were given three field strains of *Eimeria spp.* (Eimeria Pty Ltd) on d 9, and 10^8 CFU per mL of *Clostridium perfringens* strain EHE-NE18 (known to express NetB toxin, CSIRO) *per os* on d 14 and d 15. Challenge × MBM interactions were detected for body weight (BW), FCR and feed intake (FI) at d 14, 21 and 28 ($P < 0.05$) indicating that OP of MBM depressed BW and FI and increased FCR in challenged birds only. Phytase × NE interactions were detected on d 21 for BW ($P < 0.048$) and FI ($P < 0.034$) indicating the superdose of phytase increased BW and FI in unchallenged birds but decreased in challenged birds. On d 42 no 2-way or 3-way interactions were detected for BW, FCR, FI or livability. NE challenge decreased BW and FI and increased FCR ($P < 0.001$). Effects of phytase dose or OP MBM were not detectable on 42 d. These findings confirm the hypothesis that OP of MBM negatively impacts performance and increases the severity of NE incidence. A superdose of phytase was beneficial in unchallenged birds on d 21 but did not carry through to d 42. These results concur with those of Apajalahti and Vienola (2016) indicating undigested MBM protein accumulates in the hind gut where it may interact with NE infection.

Table 1 - Effect of necrotic enteritis, phytase and meat and bone meal on the performance of broilers, d 21.

2-way interactions				BW, g	FCR	Intake, g	Livability
	NE	Phytase	MBM				
NE*Phytase	-	500		904[a]	1.273	1102[a]	98
	-	5000		924[a]	1.267	1122[a]	98
	+	500		680[b]	1.476	945[b]	95
	+	5000		656[b]	1.466	904[b]	93
NE* MBM	-		Normal	925[a]	1.256[c]	1114	97
	-		OP	903[a]	1.285[c]	1111	99
	+		Normal	700[b]	1.431[b]	948	95
	+		OP	635[c]	1.511[a]	901	93
P > f							
NE				0.000	0.000	0.000	0.015
Phytase				0.839	0.357	0.445	0.613
MBM				0.000	0.000	0.082	0.866
NE × Phytase				0.048	0.827	0.034	0.400
NE × MBM				0.050	0.007	0.119	0.134
Phytase × MBM				0.992	0.138	0.530	0.400
NE × Phytase × MBM				0.534	0.239	0.911	0.241

[a,b,c] means in the same column within an interaction with different superscripts are significantly different ($P < 0.05$)

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HOW DOES *SALMONELLA* SPREAD WITHIN THE AUSTRALIAN EGG LAYER INDUSTRY?

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Summary

Globally, *Salmonella enterica subsp. enterica* is one of the most common bacterial causes of foodborne illness in humans. Contaminated food products of poultry origin, particularly egg and egg products, are frequently implicated in outbreaks of human salmonellosis. *Salmonella* Enteritidis and *Salmonella* Typhimurium are frequently involved in egg and egg product-associated foodborne outbreaks. In Australia, *Salmonella* Enteritidis infections have largely been linked with overseas travel, thus *S.* Typhimurium is currently the predominant serovar identified during local foodborne outbreaks. This paper provides an overview of *Salmonella* epidemiology on laying farms, its spread and egg contamination.

1. INTRODUCTION

Salmonella enterica subsp. enterica is the most common cause of foodborne human gastroenteritis, a disease characterised by gut inflammation and self-limiting diarrhoea in humans (Winter et al., 2010). It is estimated that gastroenteritis caused by *Salmonella* spp. accounts for 93.8 million human cases worldwide each year (Majowicz et al., 2010). Worldwide, contaminated food products of animal origin, particularly egg and egg products, are frequently implicated in outbreaks of human salmonellosis (Chousalkar and Gole, 2016). *Salmonella* Enteritidis (*S.* Enteritidis) and *Salmonella* Typhimurium (*S.* Typhimurium) phage types have dominated globally as the most common causes of human salmonellosis (Hendriksen et al., 2011). In Australia, human infections caused by *S.* Enteritidis occur largely as a consequence of overseas travel, with *S.* Typhimurium being a predominant cause of foodborne outbreaks linked to consumption of egg and egg products (Moffatt et al., 2016). Due to strict animal importing regulations and quarantine strategies of imported parent stock in Australia, *S.* Enteritidis has not been widely spread in commercial poultry flocks in Australia (Chousalkar et al., 2015). Recently, there have been some reports of *S.* Enteritidis isolation from egg and chicken meat farms in Queensland (Graham et al., 2018) and egg farms in New South Wales. Despite some reports of *S.* Enteritidis isolation from layer flocks and associated food borne outbreaks, egg products associated *S.* Typhimurium outbreaks remain the most frequently reported in Australia (OzFoodnet 2010, 2012). Numerous egg-related human *Salmonella* outbreaks have prompted significant interest amongst the public, public health authorities and industry. Thorough cooking of eggs can destroy most, if not all, bacteria present, including *Salmonella*, and cooked eggs pose low risk to human health. If egg products or food items are prepared from raw or lightly cooked egg contents, this will not destroy all *Salmonella (*if present). A rough estimate of the presence of *Salmonella* (all species, not just pathogenic) on eggs is greater than 1 in 20,000 (Arnold et al., 2014). Considering the estimated production of eggs in Australia and per capita consumption, the risk of foodborne illness in general is very low for humans consuming eggs. It has been demonstrated in a number of regions and studies that consumption of raw or lightly cooked eggs/egg preparations significantly increases the risk of human foodborne illness. This paper presents an overview of *Salmonella* epidemiology on laying farms and egg contamination.

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II. EPIDEMIOLOGICAL INVESTGATIONS AND TESTING OF FLOCKS FOR SALMONELLA

Layer hens have a common opening for the intestinal, urinary and reproductive tracts. Thus, external eggshell contamination with faecal material is often unavoidable. Although vertical transmission of *Salmonella* from bird to egg has been demonstrated (as has been shown for *S.* Enteritidis), it is generally accepted that horizontal transmission is also the most likely source of contamination of shell eggs (Gantois et al., 2009).

Currently, there is no nationwide prevalence data of *Salmonella* on egg farms. One of the major challenges in establishing prevalence is that shedding of *S.* Typhimurium from known positive hens is highly variable and can be influenced by stress experienced by hens on the farm (Gole et al., 2014a). Single time point sampling may not be sufficient to determine true prevalence. Therefore, longitudinal sampling of flocks is essential. A study conducted on commercial egg farms found that *S.* Typhimurium PT 9 was not detectable from egg contents, although the load of *Salmonella* spp on eggshell was up to log 6 CFU (Gole et al., 2014d). A *Salmonella* survey conducted on egg farms in Queensland reported that, *S.* Infantis was the most prevalent strain amongst egg layer flocks (Cox et al., 2002). Another microbiological survey conducted by New South Wales Food Authority on 49 egg farms in New South wales, showed that 20 % of the farms were positive for S. Typhimurium, whereas a survey conducted on 21 egg farms by Safe Food Queensland reported that 13.5 % of farms were positive for *S.* Typhimurium (Cuttell et al., 2014). Overall, the epidemiological investigations provide useful information on the distribution, ecology and changes in genome of *Salmonella* serovars. Conducting on-farm epidemiological investigations can be challenging due to the variable level of willingness from the farmers to participate in such studies. The epidemiological investigations concluded that the prevalence and/or shedding of *Salmonella* is influenced by flock size, flock management, shed controls, feeding practices, frequency of rodent population, production system, stage of lay, cleaning and disinfection practices adopted by a farm, level of biosecurity, exposure to wildlife vectors (birds and foxes), single aged vs multi-age flocks on farm, resting period between batches, frequency of sampling, type of samples, number of samples and timing of sampling (Denagamage et al., 2015). Epidemiological investigations (both cross sectional and epidemiological) are expensive due to high labour and *Salmonella* typing costs. In some countries, there is a requirement for more intense sampling as compared to others due to the risk of *S.* Enteritidis. In countries where *S.* Enteritidis is not endemic in commercial poultry flocks, such intensive sampling of the flock is not conducted on a regular basis. *Salmonella* positive status of faeces, egg belt and dust are significant predictors of eggshell contamination by *Salmonella* (Gole et al., 2014d). In a cage production system, the prevalence of *Salmonella* in faeces collected from the low tier cages was significantly higher compared with the samples from the high tier cages (Gole et al., 2014a) which could be attributed to the exposure of lower tiers of cages to the dust on the floor.

Birds raised in free-range production systems are potentially exposed to more environmental stressors than caged birds, including social stress and aggression, predation or thermal challenges. Free range flocks are more likely to be infected from their exposure to wildlife vectors (such as wild birds, foxes etc) (Wales et al., 2007; Wales and Davies 2011). Such wild life vectors can not only spread infections from farm to farm or to the community but also play a significant role in introducing different genomic types of *Salmonella* to the flock (Chousalkar et al., 2016). Free-range flocks are exposed to several environmental stressors, which can ultimately result in increased *Salmonella* shedding in laying hens. Stress can have an immunosuppressive effect in laying hens that could influence *Salmonella* infection and shedding. Interestingly, there was no positive correlation between faecal corticosterone metabolites and the level of *Salmonella* shedding (Gole et al., 2017).

It is difficult to predict or determine whether eggs produced by free range and or free range organic production systems are “high risk” compared to caged production systems as the level and prevalence of *Salmonella* in the flock or on the farm is attributed to individual flock and/or farm management. More recently, a longitudinal study conducted on free-range farms from day old to end of the commercial life span indicated that birds are most likely to be exposed with *Salmonella* during production (McWhorter and Chousalkar, 2019).

S. Typhimurium and other serovars are able to survive/persist in the shed environment (such as in dust) so regular cleaning and or removal of dust from shed is important. Use of vaccination in multi-age flocks or single aged flock is “not an ultimate intervention” for reduction of *Salmonella* Typhimurium because of the complexities involved in achieving control, such as the efficacy of cleaning of sheds, the lack of resting periods between batches and the possible carryover of contamination from existing flocks. Hence implementation of more than one or several interventions strategies is essential (McWhorter and Chousalkar, 2018; Sharma et al., 2018). Throughout the life span of a laying hen, *Salmonella* can be introduced to a farm through various vehicles such as rodents, contaminated egg trays, people movement, and contaminated trucks, introduction of infected flock, contaminated feed or contaminated equipment (Chousalkar and Gole, 2016). In Australia, the number of backyard chickens is also on the rise. The biosecurity standards of backyard chickens is highly variable and *Salmonella* infected flocks could pose a significant risk to commercial flocks (Manning et al., 2015). Hence, regular cleaning, disinfection of equipment and a continuous review of on-farm biosecurity standards is critical.

III. SALMONELLA PENETRATION, SURVIVAL IN AND ON EGGS

Egg and eggshell quality could play a vital role in trans-shell penetration of *Salmonella* spp. A good quality eggshell significantly protects the internal contents from bacterial penetration. A cracked or damaged egg encourages bacteria to move across the eggshell, which may result in food poisoning. Eggs possess a cuticle, which acts as the first line of defence against bacteria (Gole et al., 2014c). This covers the external surface of the eggshell and functions to close the eggshell pores soon after lay to decrease bacterial penetration. In warm, freshly laid eggs, the cuticle does not mature for a period of time after lay, therefore leaving some pores open for *Salmonella* penetration (Miyamoto et al., 1998). Several surveys have been conducted on eggs to study the level of *Salmonella* contamination in and on retail or first grade eggs. The *Salmonella* contamination of table eggs was reviewed by (Martelli and Davies, 2012). The review concluded that *S.* Typhimurium is often not isolated from eggs compared with *S.* Enteritidis. This finding was later confirmed during field epidemiological investigation (Gole et al., 2014d) where eggs laid by *S.* Typhimurium flocks were not always contaminated. Egg-based *Salmonella* surveys provide some useful information on distribution of *Salmonella* serovars in different parts of the world, but the results of surveys are highly variable. Moreover, egg-based *Salmonella* surveys do not necessarily reflect the level or prevalence of *Salmonella* contamination at farm or bird level. Experimental investigations also reported a lack of correlation between egg contamination and duration of *Salmonella* shedding in faeces (could be interpreted as individual bird infection) (Gast et al., 2005; Pande et al., 2016a). The egg handling practices on or off farm could also influence the level of *Salmonella* contamination on eggshell.

Eggs have natural defence barriers for protection against the bacterial penetration. Egg albumen has many antibacterial properties but *S.* Enteritidis phage types have the ability to replicate in the oviduct, contaminate the developing egg, multiply in the albumen (Gantois *et al.* 2009) and several relevant genes associated with survival of S. Enteritidis in egg white have been identified (Clavijo et al., 2006). It has been hypothesised that *S.* Enteritidis uses the stress

induced survival mechanism for survival in the egg white and colonise the oviduct (Van Immerseel, 2010). It has also been demonstrated that the virulence properties of *S.* Enteritidis are unaffected by the hostile environment within the egg albumen (Baron et al., 2004). *In-vitro* studies found that *S.* Typhimurium phage types have the ability to survive in the egg albumen, egg yolk and on the eggshell surface (Gantois et al., 2008; Gole et al., 2014b) and *S.* Typhimurium isolates possess genes associated with the survival of *Salmonella* in egg albumen (McWhorter et al., 2015). However, it is not clear whether the hostile environment within the egg albumen affects the virulence of S. Typhimurium. It is well established that penetration of *Salmonella* serovars is influenced by temperature, egg and eggshell quality, bacterial strain, pH and moisture (De Reu et al., 2006). *In-vivo* studies on the mechanism of egg contamination by *S.* Typhimurium provided variable results and this variation could be attributed to the dose, phage type, timing and route of infection (Wales and Davies, 2011). *S.* Typhimurium was localised in reproductive organs, egg internal contents tested negative (Okamura et al., 2010; Pande et al., 2016a), whereas another study found that egg internal contents were contaminated when hens were infected with S. Typhimurium by the aerosol route (Leach et al., 1999). *S.* Typhimurium has the ability to form a viable but non-culturable state (VBNC) and retain its invasive ability (Passerat et al., 2009). It could be hypothesised that some *S.* Typhimurium phage types enter this VBNC state up on exposure to fresh egg white and further studies are required to investigate this hypothesis. The presence of *Salmonella* on the eggshell underlines the importance of proper handling of eggs in the food industry as well as in the kitchen environment to avoid cross contamination of other food items.

IV. WHERE TO FROM HERE?

An increase in production and consumption of eggs and related *Salmonella* outbreaks in the Australia indicates that egg related *Salmonella* is likely to remain a concern for public health. *Salmonella* diagnostics, reporting and surveillance systems have improved over the years and will continue to improve in the years to come. Given the number of different emerging *Salmonella* serovars, a regular review of *Salmonella* control strategies from farm to fork is required. On farm epidemiological surveillance is essential to control and monitor the circulating *Salmonella* serovars and respective changes in genotype. Several other serovars other than *S.* Enteritidis and *S.* Typhimurium have been periodically implicated in egg related *Salmonella* outbreaks (Glass et al., 2016). Given the ability of *Salmonella* serovars to transfer or acquire genes via horizontal gene transfer with plasmids, transposons, and phages *(*Foley et al., 2013), the evolution of further virulent strains is not unexpected. The intervention practices such as vaccination and use of egg washing can reduce the load of *Salmonella* spp on the egg but not eliminate it. *Salmonella* serovars are not only able to survive in harsh environment in production and the supply chain but are able to quickly multiply if favourable environmental conditions are available. The implementation of regulations and policies for control of *Salmonella* on-farm has put onus on egg farmers and processors but end users also carry a significant responsibility of safe handling of eggs and egg products. Cultural diversity has contributed to a far wider selection of food, incorporating a greater range of raw foods of animal origin into our diet. The efficacy of messages such as 'cook eggs thoroughly' or 'wash your hands' will depend both on the ability to change consumer behaviour as well as where the risk can best be mitigated (Luber, 2009). It is also important to note that the food handling behaviour could be influenced by sex, income status and age of an individual (Patil et al., 2005). There is sufficient evidence to suggest that *Salmonella* spp are able to survive and persist on eggshells at varied levels. While egg handling procedures coupled with kitchen cleanliness limit the possibility of infection, only 10^2 CFU (colony forming units) of pathogenic strains of *Salmonella* are required to cause disease in humans (Fabrega and Vila, 2013). *Salmonella*

serovars also have the ability to form biofilms on egg shells at ambient temperature (Pande et al., 2016b). Biofilms on the eggshell surface could act as a vehicle for contaminating food products, further spreading the pathogenic bacteria to other hosts risking public health. Continuing research is required to refine egg handling and hygiene practices.

To improve the reporting system, the general public also need to be more vigilant and aware about the onset of clinical symptoms, and the risk of spreading infection to very young, old or immunocompromised people. Such awareness could be implemented through general physicians/medical practitioners. Continuous dialogue between egg producers, regulators and researchers is critical to develop and implement the innovative *Salmonella* control strategies.

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SELECTIVE REMOVAL OF *SALMONELLA* FROM BROILERS USING A NOVEL TECHNOLOGY

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Summary

A new CRISPR-based patented technology has been developed that promotes the selective removal of specific unwanted bacteria by inducing self-destruction. This technology, designed to remove all *Salmonella* serovars and introduced into a vector *Escherichia coli* probiotic for delivery, was tested in the current trial in broilers challenged by the introduction of *Salmonella enteritidis*-positive seeder birds at 5 days of age. Three groups each of 45 as-hatched Ross 308 chicks were used, with one group as a Control and the other groups supplied via drinking water with either the *E. coli* vector probiotic alone or with an anti-*Salmonella* plasmid included (Guided Biotics™). Caecal samples (15 per group) were taken at day 12 (7 days after introduction of three *Salmonella* colonized seeder birds per group) for *Salmonella* counts (both direct and enhanced), and bird liveweight was recorded at 42 day. All 30 birds tested in the Control and vector-only groups were *Salmonella* positive at day 12 with the enhanced method, with caecal counts of 500-4,000,000 CFU/g, while 22 were positive by the direct count method. Inclusion of the vector + plasmid combination in the drinking water resulted in no detection of *Salmonella* in any birds using direct counts, whilst 8 of the 15 birds tested *Salmonella* free with enhanced counts. This combination also reduced the mean *Salmonella* counts by approximately log-3 ($P<0.001$). The birds in the vector + plasmid group were 15% heavier ($P=0.02$) than the Control and vector-only groups. This trial established the ability of this Guided Biotics™ technology to selectively remove specific bacteria from the bird gut.

I. INTRODUCTION

Over the past few decades the meat, egg and milk sectors have faced the need to reduce the routine use of antibiotics in animal production, and the high incidence of food poisoning associated with animal product consumption. Approximately 130,000 tonnes of antibiotics were used in 2013 worldwide, with 75% of this in animals (Hughes, 2019). Up to 90% of these antibiotics can be excreted into the environment via urine and faeces, and approximately 400 resistance markers against 25 antibiotics can be found in chick caecal bacteria (Van Boeckel *et al*, 2019). Globally, around 700,000 human deaths per annum are attributed to antibiotic resistance and this is predicted to increase to 10 million by 2050 (FAO, 2019). With rising concern about the development of antibiotic resistance in human health, regulators, consumers and retailers have led the drive to reduce the sub-therapeutic use of antibiotics in animal feeds to zero. Endemic disease is re-emerging, adding costs to animal production systems and driving the need for alternative non-antibiotic interventions.

Food poisoning continues to be a problem across the world, with salmonellosis cases now increasing in many countries. Non-typhoidal salmonellosis is reported to cause over one million infections, 19000 hospitalizations and over 400 deaths annually in the US (Forkus *et al*, 2017), with some *Salmonella* serovars in food showing antibiotic resistance. Although salmonellosis incidents are traditionally relatively low in Australia, recent egg-associated outbreaks have brought this back to the attention of the regulators and consumers.

It is now possible to cause a targeted bacterium to self-destruct through the use of CRISPR, the biological sequences that make up the bacterial immune system (Hamilton et al.,

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2019). This technology is extremely precise, such that it can target a specific bacterium or a defined range of bacteria. This means that, unlike many antibiotics, it can be used to remove only the unwanted bacteria in the animal gut microbiome and leave beneficial gut flora unchanged, potentially enhancing the well-being of the animal. One way to induce bacteria in the animal gut to self-destruct is to introduce a suitable plasmid into the target organism(s) through conjugation via a probiotic included in the feed or drinking water. The current trial looks at the ability of this technology, named Guided Biotics™, to reduce *Salmonella* colonization in challenged broilers.

II. METHOD

A non-pathogenic *Escherichia coli* strain was used as the vector in this trial, and was loaded with a plasmid including a CAS sequence and 3 target sequences specific to all *Salmonella* serovars (Guided Biotics™). Ross 308 as-hatched birds (165) were obtained on day of hatch and housed under controlled biosecure conditions, with access to water and standard commercial rations *ad libitum*. Birds were dosed continually from day 1 with either:

1. No addition to water (45 birds)
2. Unmodified *E. coli* vector at 10^8 cfu/ml drinking water (45 birds)
3. Anti-*Salmonella* Guided Biotics™ at 10^8 cfu/ml drinking water (45 birds)

In parallel, a group of 30 birds was dosed orally with 0.5 ml 10^5 CFU/mL *Salmonella Enteritidis* strain FS26 on day 1. Birds were checked for *Salmonella* colonisation at day 3 by cloacal swab (ISO 6579-1:2017). On day 5, three verified *Salmonella*-colonised birds (seeder birds, with $>10^5$ CFU/g in swabs) were marked and added to each of the test groups.
Fifteen non-seeder birds from each group were euthanased on day 12 (7 days post-mixing with seeder birds) and caecal contents were counted for *Salmonella* using both direct and enhanced methods. Caecal samples were serially diluted in PBS before plating onto XLD agar for direct counts, whilst for enhanced counts the samples were first incubated in Selenite Cystine broth for 18 hrs at 41^{o}C before plating and counting (ISO 6579-1:2017). For the purpose of data transformation, samples negative in either method were allocated a count of 1 CFU/g, while those negative in direct counts but positive in the enhanced method were allocated 500 CFU/g. Body weights of the remaining birds were monitored at day 42. Counts and weights were log transformed and statistical analysis conducted using GraphPad Prism. Data were assessed for normality of distribution using a D'Agostino and Pearson omnibus normality test and non-normal were analysed using a Kruskall-Wallis test with Dunn's multiple comparison test post hoc. Differences were analysed using Fisher's exact test.

III. RESULTS

All birds in the seeder group showed cloacal *Salmonella* counts of $>10^5$ CFU/g by day 3. By day 12 (7 days post introduction of seeder birds to test groups) all birds in the Control and *E. coli* vector-only groups were positive using the enhanced counts method, exhibiting caecal counts of 500-4,000,000 CFU/g (Table 1). Twenty two of these 30 birds were also positive with direct counts. However, when the anti-*Salmonella* Guided Biotics™ was added to the drinking water, *Salmonella* was not detected in any birds with the direct method, and only 8 of the 15 birds tested were positive with enhanced counts. The Guided Biotics™ treatment reduced ($P < 0.001$) mean *Salmonella* counts by approximately log-3 (from log 4.12 to log 1.26, equivalent to 14,200 CFU/g to 18 CFU/g) and also improved 42-day liveweight by 15% ($P = 0.02$; Figure 1).

Table 1 - Influence of the *E. coli* vector alone or Guided Biotics™ with an anti-*Salmonella* plasmid on caecal *Salmonella* counts (log^{10} CFU/g, enhanced counts method) in 12-day old *Salmonella*-challenged broilers.

	Control	*E. coli* vector only	Guided Biotics™
Mean *Salmonella* count (log^{10} CFU/g)	4.12[a]	4.74[a]	1.26[b]
Median *Salmonella* count (log^{10} CFU/g)	3.30	4.95	ND
Maximum *Salmonella* count (log^{10} CFU/g)	6.60	6.60	2.70
Minimum *Salmonella* counts (log^{10} CFU/g)	2.70	2.70	ND

[a-b]P < 0.001
ND – not detected

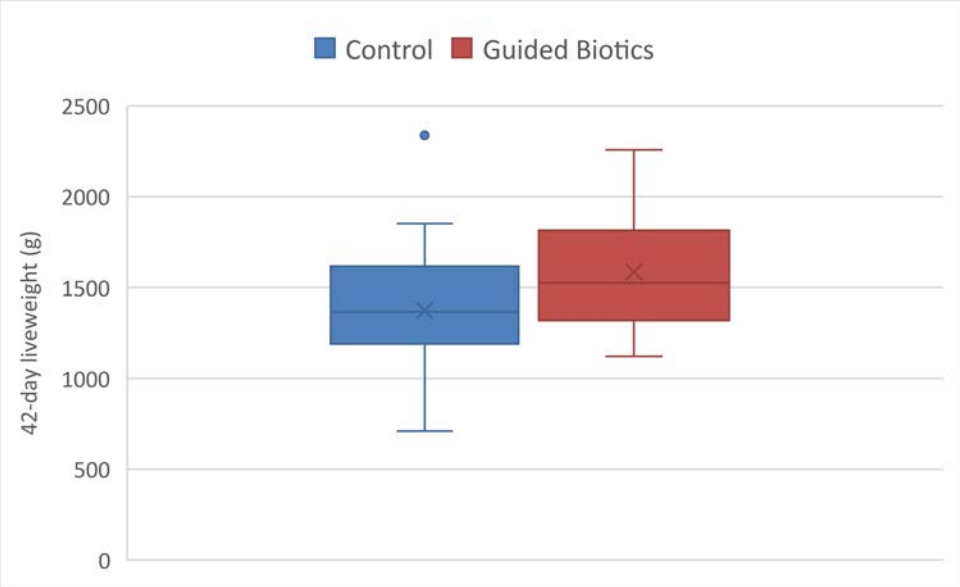

Figure 1 - Influence of Guided Biotics™ on bird liveweight at 42 days of age (g).

IV. DISCUSSION

The challenge method employed in this study is consistent with that often use in *Salmonella* vaccine tests and may be regarded as severe (Cooper et al., 1994). All seeder birds were infected when introduced into the test pens, and the *Salmonella* shed to in-contact birds would be expected to be highly infective. This was confirmed by the universally high caecal counts in all Control birds 7 days after seeded-bird introduction. Conversely, the Guided Biotics™, delivered by conjugation in the digestive tract, was able to stop *Salmonella* colonization in 8 out of 15 (53%) of the test birds. The average *Salmonella* count in caecal digesta was also reduced by approximately log-3 (thousand-fold) and the maximum *Salmonella* count lowered from 4 million CFU/g in Control birds to 500 CFU/g in Guided Biotics™ treated. The 15% increase in liveweight of birds fed the Guided Biotics™ relative to the Control birds further indicates the severity of the *Salmonella* challenge employed in this trial. The lack of any effect of the *E. coli* vector on colonization confirms that the Guided Biotics™ plasmid was essential for *Salmonella* reduction.

This initial trial establishes the capability of Guided Biotics™ technology to specifically remove unwanted bacteria, in this case a single *Salmonella* serovar. The tested Guided Biotic™ is designed to target all known 2400 Salmonella serovars, and laboratory trials have established efficacy across the main serovars involved in human food poisoning. Ongoing laboratory tests have also indicated that solutions for other unwanted bacteria, such as *Clostridium perfringens* and Avian Pathogenic *E. coli*, are feasible. Furthermore, because the

design of the targeting is specific, tests have confirmed that off-target killing of desirable or commensal bacteria can be avoided. It is clear that this Guided Biotics™ technology has the potential to make a substantial contribution to the replacement of antibiotics in poultry production, reduce zoonosis incidents and maintain bird performance in antibiotic-free diets.

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PATHOGENESIS OF EGG INFECTIONS BY *SALMONELLA* AND THE IMPLEMENTATION OF PREVENTIVE MEASURES

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Summary

Salmonella is one of the most important zoonotic bacterial pathogens, mainly caused by consumption of contaminated food products, and is of global importance. Strains from the serotypes Enteritidis and Typhimurium are most important for human food poisoning, and the former are highly associated with egg consumption. *Salmonella* is an invasive bacterial genus and colonizes the gut of chickens, and systemically spreads to internal organs, including the reproductive tract, potentially leading to egg contamination. Monitoring programs are essential to assess the prevalence of infected flocks and detect changes in prevalence and are important to evaluate the efficacy of control methods and programs. Live and attenuated vaccines are in use and these give partial protection against intestinal and internal organ colonization, and egg contamination. Early protection has been demonstrated after primary live vaccine administration early post-hatch but this effect is serotype-specific, while cross-protection between some serotypes has been demonstrated after booster immunisations. Vaccines can only be efficient when biosecurity is optimal. A variety of nutritional strategies can be used to reduce *Salmonella* colonization, also for broilers. It is a utopia to eradicate *Salmonella* from chickens and the environment, but one should try to aim for appropriate levels of protection, and thus low flock prevalence and within-flock prevalence, and low individual colonization levels.

I. EPIDEMIOLOGY OF *SALMONELLA* INFECTIONS

Most *Salmonella* strains belong to non-host specific or broad-host range serotypes, and thus can colonize the gut of many animal species, including humans. In contrast to host-specific serotypes that cause septicemia and severe disease (typhoidal serotypes), the broad-host range serotypes are asymptomatically colonising the host in most cases, but can cause diarrhoea when high numbers of bacteria are orally take up at once, as is the case in human food poisoning (non-typhoidal serotypes). *Salmonella* is one of the most important zoonotic bacterial pathogens, mainly caused by consumption of contaminated food products, and is of global importance. The global burden of gastroenteritis due to *Salmonella* has been studied by various authors. Majowicz et al. (2006) estimated the global number of *Salmonella* cases to be around 1600 million cases per year. An estimation of the global and regional disease burden by the World Health Organization shows non-typhoidal *Salmonella* to cause highest numbers of disability-adjusted life years (DALYs) of all foodborne pathogens (Kirk et al., 2015). DALYs are defined as the sum of the years of life lost due to premature mortality in the population and the years lost due to disability for people living with the health condition or its consequences. While many serotypes can be transmitted to humans due to contaminated meat products (mainly chicken and pork, but also cattle, fish and other sources), eggs are the main food vehicle for human infections. As an example, in the European Union in 2017, more than 50% of the human cases were egg-derived (37% egg-derived, 17% derived from bakery products). The other 50% was derived from various sources such as mixed food products (13%), meat products (8%), poultry meat (2.2%), porcine meat (4.5%), and to a lesser extent cheese, dairy, vegetables and fish (EU data, 2017). Interestingly, about 50% of all cases are caused by strains

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of the *Salmonella* serotype Enteritidis, and 25% by serotype Typhimurium strains (of which 8% are monophasic variant strains). The other 25% is caused by a variety of serotypes, including *Salmonella* Infantis (2.5%) (EU data, 2017). It is striking that, for many of the serotypes that are important for human food poisoning, there is an association with specific food sources. *Salmonella* Enteritidis is of particular importance because it can spread to the reproductive tract and contaminate eggs. A worldwide egg-associated salmonellosis pandemic started in the '70s and is currently fading away in many countries, thanks to huge efforts of policy makers and the poultry industry. This pandemic has been specifically caused by the serotype Enteritidis. Due to its preferential association with hen eggs, combined with the way humans tend to store (room temperature), handle and eat (uncooked) eggs, *Salmonella* Enteritidis had and still has a major impact on human health. Strains from the serotype Typhimurium are associated with many sources, including porcine, cattle, turkey and chicken meat, but also eggs. Strains from the monophasic variant of serotype Typhimurium are specifically associated with food poisoning cases and outbreaks after porcine meat consumption. In addition to the human infections caused by the 2 predominant serotypes, Enteritidis and Typhimurium, also many other serotypes can cause human gastroenteritis. These are mainly derived from meat sources, and the nature of the serotypes depends on the geographical location, and changes during time. Serotypes such as Hadar, Infantis, Paratyphi B, Heidelberg, Minnesota and many others can be derived from poultry meat. Strains from *Salmonella* Newport associate with turkey meat (EU data, 2017). A specific trend is the spread of clonal lineages of certain serotypes that are often multidrug resistant, in poultry. A meta-analysis of Ferrari et al. (2019) on the global epidemiology of *Salmonella* shows that there are some serotypes that are colonizing poultry worldwide (Enteritidis, Typhimurium, Infantis, Hadar, Kentucky), while there are serotypes that are specifically associated with certain regions (eg. Heidelberg in the Americas, Mbandaka in Europe). Oceania is an exception as Enteritidis, Hadar and Kentucky are not an issue (recently Enteritidis entered however), while serotypes such as Sofia and Kiambu are specific for this region (Ferrari et al., 2019).

II. THE PATHOGENESIS OF *SALMONELLA* INFECTIONS IN POULTRY

Chickens usually are infected by oral uptake of bacteria from the environment. *Salmonella* bacteria are able to survive gastric acidity and can pass the stomach to reach the intestinal tract of the animal. The caeca are the predominant colonisation sites. The bacteria can adhere to and invade caecal epithelial cells, by rearranging the actin cytoskeleton of the epithelial cells in such a way that bacteria are engulfed by ruffles on the host cell membrane, resulting in uptake by the epithelial cell. This process of invasion is mediated by a type three secretion system, encoded by genes of the *Salmonella* pathogenicity island I, and is essential for caecal colonisation (Bohez et al., 2006). Immune cells are attracted to the gut wall and the macrophages may take up bacteria penetrating through the caecal mucosa. This is the start of the systemic phase of the infection as *Salmonella* bacteria can survive within and replicate in these macrophages. These cells spread the bacteria to the internal organs, such as liver, spleen, ovary and oviduct (Bohez et al., 2008), where the bacteria can be found in large numbers. All this is serotype and strain dependent, and some strains are more invasive than others while others are less or not efficient in persistent caecal or organ colonisation. Shedding can occur intermittently. Contamination of poultry meat can thus be caused by contamination in the slaughterhouse when faecal material or gut content (or internal organ material) is contaminating the carcasses during the slaughter process (for example evisceration, defeathering). Eggs can be contaminated either externally (on the shell) or internally (Gantois et al., 2009). Shell contamination is caused by contamination during or after lay, because faecal material or *Salmonella* bacteria present in the environment contaminate the outer shell. Internal

egg contamination can be caused by *Salmonella* bacteria that are transported through the eggshell after shell contamination. In addition, internal egg contamination can be caused by *Salmonella* bacteria that are incorporated in the forming egg during passage in the oviduct. *Salmonella* can colonise the oviduct after systemic spread and thus contaminate the egg components, depending on the site of colonisation (magnum, egg white; isthmus, shell membrane). While all *Salmonella* serotypes, to a greater or lesser extent depending on the serotype and strain, are able to colonize the gut and internal organs, *Salmonella* Enteritidis is far more capable of persisting in the oviduct as compared to other serotypes (Gantois et al., 2008). In addition, *Salmonella* Enteritidis strains have been shown to be superior in egg white survival (De Vylder et al., 2013). The lipopolysaccharide (LPS) structure and multi drug resistance efflux pumps have been shown to be involved (Raspoet et al., 2014, 2019). The egg white is very antibacterial (high pH, a variety of antimicrobial proteins and peptides) and is capable of killing most bacteria, including most *Salmonella* serotypes, but Enteritidis strains are rather resistant. These characteristics of *Salmonella* Enteritidis explain its success in contaminating and infecting humans. In addition, *Salmonella* Enteritidis is not growing in egg white, but only staying alive and thus no sensory or visual changes occur in the contaminated eggs. Consumers are thus not alerted.

III. MONITORING AND CONTROL PROGRAMS

Monitoring programs are of utmost importance in a global strategy of controlling *Salmonella*, because they assess the prevalence of infected flocks (or even the within-flock prevalence, depending on the method used) and detect changes in prevalence, as well as the serotype distribution, clonal spread, etc. They can also be used to evaluate the efficacy of control methods and programs. Periodic testing using bacteriological detection methods is the most widely used method, but serological methods can also be of value. Bacteriological testing methods are often based on excretion of *Salmonella*, and thus have inherent problems with sensitivity because infected chickens shed *Salmonella* intermittently. This can partly be overcome by using mixed faecal samples so that faecal material of many animals is analysed. The within-flock prevalence can however also be low and, if only a low number of animals shed *Salmonella*, this method will most likely often not detect these infections. Mostly, if a number of samples is analysed and 1 sample is positive, the flock is considered to be *Salmonella* positive. This positivity is thus not giving information about the actual number of infected animals and the colonization level in the animals. The analytical methods used to detect *Salmonella* are based on enrichment of the samples for *Salmonella* and plating of the enriched material on different selective media, often followed by serotype identification. The frequency of the sampling depends on the animal type (breeders, layers, broilers) and the production stage (e.g. pullets vs layers). Often the frequency is higher for breeders as compared to layers, because these animals can contaminate the whole production chain by vertical transmission. As an example, under EU legislation (2160/2003), sampling and detection of all *Salmonella* serotypes with public health significance should be done according to the following schemes: (a) breeding flocks: day-old, 4 weeks, 2 weeks before transport to the laying unit and every 2 weeks during lay; (b) laying hens : day-old, 2 weeks before transport to the laying unit and every 15 weeks during lay; (c) broilers : before transport to the slaughterhouse. In addition to bacteriological detection, also antibody responses in serum can be used to monitor the *Salmonella* status of a flock. Antibody detection tests are available in ELISAs and typically detect either O antigens (LPS) or H antigens (flagelllin). While bacteriological detection methods have a higher chance of detecting positive animals in the early period post-infection due to higher excretion, serological tests can detect positive animals a long time post-infection and do not detect antibodies in the early post-infection period due to the dynamics of antibody

production after infection. Not all animals, however, generate an efficient antibody response, and also here the number of samples to be taken is not easy to calculate, and this depends on the actual minimal to be detected within-flock prevalence and the accuracy that is defined beforehand. Both methods thus have advantages and disadvantages.

Although control tools are available to reduce *Salmonella* colonisation, there needs to be a general strategy on the control methods to be used and defining the situations in which specific measures need to be implemented, but also on the actual consequences of finding *Salmonella* positive samples. For example, for breeding flocks this can mean that the hens need to be eradicated when certain *Salmonella* serotypes are detected. For layers and broilers, the finding of certain serotypes could imply that the eggs or meat have to be treated in a way that kills the bacteria before the food is marketed. For example, in the EU (Regulation 2160/2003) in case of an infection with *Salmonella* Enteritidis or Typhimurium in breeding flocks, non-incubated hatching eggs should be destroyed or used for human consumption following treatment in a manner that guarantees the elimination of *Salmonella* Enteritidis and Typhimurium. All birds from these flocks must be slaughtered or destroyed, even the day-old chicks. Eggs derived from these birds that are still present in a hatchery, also have to be destroyed or treated as described above. Another specific requirement is that eggs must not be used for human consumption as fresh table eggs unless they originate from a commercial layer flock subject to a national control programme. Moreover, eggs originating from flocks with unknown health status, suspected of being infected or from infected flocks may only be used for human consumption if treated in a manner that guarantees the elimination of all *Salmonella* serotypes with public health significance. The use of control methods on the farms can be made obligatory, depending on the *Salmonella* status of the flocks, or even the *Salmonella* status of the flocks in a region.

IV. VACCINATION TO REDUCE *SALMONELLA*

A lot of experimental vaccines have been produced for chickens, and also a variety of commercial vaccines are available on the market. These comprise both live and inactivated vaccines. The currently available live vaccines are produced by chemical mutagenesis or are selected on culture media as slow growing natural mutants (metabolic drift mutants). In general, it is believed that live vaccines induce better protection because they stimulate both cell-mediated responses and antibody responses, while inactivated vaccines mainly induce antibody production, but both methods are in use, singly or in combination in vaccination regimens. Triple dose vaccination schemes are common for layers and breeders, and also combinations of live and inactivated vaccines are given. Live vaccines are mostly administered in the drinking water (or using a coarse spray) and inactivated vaccines need to be administered parenterally. Autologous vaccines are used in some countries, made by killing a strain isolated from the flock where the vaccine is administered. Cross-protection is shown to be occur but it is believed that intra-serotype and intra-serogroup protection is more pronounced. For example, Eeckhaut et al. (2019) showed that live Enteritidis vaccines significantly reduce *Salmonella* Infantis colonization in layers.

Vaccines have been used extensively in laying hens and should a) reduce or prevent the intestinal colonisation resulting in reduced faecal shedding and thus egg shell contamination and b) prevent systemic infection resulting in a decreased colonisation of the reproductive tissues, in this way reducing internal egg contamination. Inactivated vaccines are often used in parent flocks. Parenteral administration of inactivated *Salmonella* vaccines to breeder birds will induce a strong production of antibodies. These antibodies will be transferred to the progeny. The maternally transferred antibodies persist for a few weeks but, although there seems to be some protective effect against disease in the early post-hatch period, there is little

effect on intestinal colonisation by challenge strains (Methner and Steinbach, 1997; Methner et al., 1994). There is a report on the efficacy of inactivated vaccines in prevention of egg contamination in layers (Woodward et al., 2002). Gantois et al. (2006) showed that oral vaccination with live vaccines at day 1, week 4 and week 16 decreased internal organ colonisation, including reproductive tract colonisation, and egg contamination. Although it is very difficult to prove reduction of egg contamination following vaccination under field conditions owing to the low and variable percentage of contaminated eggs laid, a European baseline study showed that vaccinated layer flocks were less frequently contaminated by *Salmonella* as compared to non-vaccinated flocks (4% vs 12%). In theory, an ideal live vaccine strain should possess following characteristics (Van Immerseel et al., 2005):

- Induce a high degree of protection against systemic and intestinal infection
- Protect against a variety of important serovars (serogroups)
- Show adequate attenuation for poultry, other animal species, humans and the environment
- Be easy to administer without animal welfare issues
- The inactivated and live vaccines should not affect growth of the animal
- Vaccine strains should not be resistant to antibiotics (or contain resistance genes)
- Vaccines have markers facilitating the differentiation from *Salmonella* wild-type strains
- Application of vaccines should not interfere with *Salmonella* detection methods
- Humoral antibody response after vaccination should be distinguishable from a *Salmonella* wild-type response to allow the use of serological detection methods

Multiple scientific groups have reported a phenomenon, in which oral administration of *Salmonella* wild type and attenuated strains can confer resistance to infection by a virulent *Salmonella* challenge strain within 24 h of administration. This ‘competitive exclusion’-like phenomenon is called colonization-inhibition. These data suggest that it might be possible to administer live *Salmonella* vaccine strains to newly hatched chicks such that they would colonize the gut extensively and very rapidly, inducing a profound resistance to colonization by other *Salmonella* strains of epidemiological significance, which may be present in the poultry house or may also have arisen from the hatchery (Van Immerseel et al., 2005). Colonisation of the gut by the colonisation-inhibition strains would prevent gut colonisation by virulent strains, while invasion in the gut tissue would evoke an inflammatory response that would prevent invasion to the internal organs by virulent strains. This means that live vaccines can thus also be used in broilers to control gut colonisation and shedding. An issue is to administer the strains as early post hatch as possible to the birds; this is not ideal using drinking water applications but can be done using coarse sprays (De Cort *et al.*, 2014).

It is difficult to speculate about the nature of future vaccines but good methods are available to rationally design live vaccines that have defined mutations so that both detection methods and safety aspects are highly controlled. These are, however, genetically modified organisms and their use is still under debate although some are already marketed. Many research groups have designed genetically modified live vaccines with a very good safety and efficacy profile, and with markers that are differentiating the strains and the serological response from wild type strains and serum responses, respectively. In relation to emerging phenotypes and the variety of *Salmonella* phenotypes in broilers, developing vaccines against other serotypes can become a need, but the registration process is long, hampering development of these vaccines.

To be complete, one needs to mention that, in addition to vaccines, other methods are available and a multiple hurdle approach is needed. Biosecurity is crucial, and it is evident that rodent and insect control and general hygienic and biosecurity measures are a prerequisite for keeping *Salmonella* out of the farms. In addition, many drinking water and feed additives are being used, including organic acids such as butyrate, prebiotics, probiotics and phytochemicals,

amongst others. This is not within the scope of this paper but reviews can be consulted: Van Immerseel et al. (2002), Micchiche et al., (2018), Clavijo and Flores (2018) and many more. It is a utopia to eradicate *Salmonella* from chickens and the environment, but one should try to aim for appropriate levels of protection, and thus low flock prevalence and within-flock prevalence, and low individual colonization levels.

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VALIDATION OF A RADIO FREQUENCY IDENTIFICATION (RFID) SYSTEM FOR AVIARY SYSTEMS

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Radio Frequency Identification (RFID) technology has been used for animal behaviour and welfare research, to monitor animal location and resource utilization. The aim of this study was to develop and validate an in-house custom built ultra-high frequency (UHF) RFID system to be used on a commercial free-range egg farm with three-tier aviaries. The system was designed and constructed to detect hens in certain areas (feeders, nest boxes, range) within the housing system. Briefly, Speedway R420 RFID isotropic tag readers (Impinj, Seattle, WA, USA), Monza R6 UHF RFID Tags, (Impinj, Seattle, WA, USA) and Clear Stream RFID software developed by Portable Technology Solutions (Calverton, New York, USA) were used to identify and track individual hens. The antennae were tested at a frequency of 900 mHz using 60 randomly selected RFID leg bands for signal strength with Received Strength Indicator (RSSI) values at varying vertical and horizontal distance (0, 25, 55, 85, 120, 155, 200, 300, 400 and 500 mm). Furthermore, 18 randomly selected hens on-farm were equipped with 2 tags each (one on the left leg, one on the right leg) and the number of events and time duration of these tags were observed for 30 consecutive days while being exposed to 27 antennae through the RFID system. The data from the tags were fitted into a general linear model using JMP, version 14, SAS Institute Inc., Cary, NC, 1989-2019.

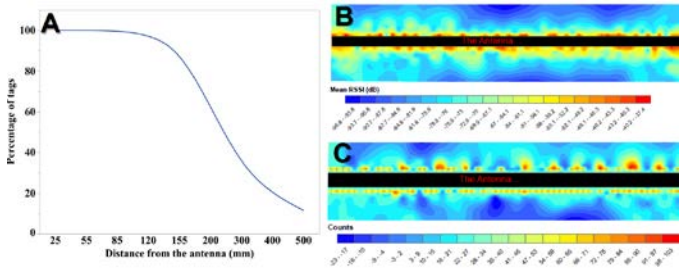

Figure 1 - (A) Inverse square law curve fit of the tag read accuracy of the antennae in relation to the distance from the antenna. (B) Bivariate density plot of the average signal strength (RSSI)/ second and the distance from isotropic antennae of the tested tags. (C) Bivariate density plot of the count read and the distance from isotropic antennae of the tested tags.

The highest signal strength of -37.4 dB was recorded at 25 mm from the antenna. At this distance, 100% of RFID tags were recorded (Figure 1). At a distance of 500 mm, no significant signal strength could be recorded ($P > 0.05$). There was a significant detection reliability of two tags attached to one hen (one tag in each leg) in the number of events ($R^2 = 0.66$; $P = 0.0001$) and time duration ($R^2 = 0.53$; $P = 0.0001$). This was most likely because both legs of a hen are rarely at exactly the same distance from the antenna, due to the orientation of the RFID microchip, or due to potential signal interference with the metal construction of the aviary system. In conclusion, the system was effective at reading the tags at a distance of 155mm with 90% accuracy and the results show potential for the system to be used indoors.

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THE IMPACT OF PERCH SPACE ON THE ACTIVITY, BEHAVIOUR AND LEG HEALTH OF MEAT CHICKENS

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Compromised leg health is a welfare and economic concern for meat chickens. Enrichment of the production environment via the provision of perches has been a focal point of research into reducing the prevalence of leg disorders in recent years. Despite inconsistent evidence of the effect of perches (Groves and Muir 2013; Phibbs et al. 2019), they are provided in almost two thirds of the Australian meat chicken industry, with 2.7 m perch/1000 birds required under the RSPCA Approved Farming Scheme (RSPCA Australia, 2019). This study aimed to evaluate whether the quantity of perch space provided impacts bird activity, behaviour and leg strength.

Day-old mixed-sex Cobb 500 chicks were randomly allocated across four perch space/1000 bird treatments viz: 2.7 m (A); 5.4 m (B); 47.6 m (C) and 0 m (D – control). Perches were an A-frame wooden design, with treatments A and B having one perch, 15 cm off the ground and treatment C having two perches, 15 cm and 30 cm off the ground. All perches were squared and 42 mm wide. The study took place in deep litter pens each housing 84 birds, stocking density 28kg/m^2 at 42 days of age (d), in a controlled environment shed, with six replicates/treatment. Overall feed consumption and bird weight from 0 d to 42 d were measured. Litter pH and moisture were measured biweekly throughout study. Bird activity was observed at five consistent time points every second day, starting at 1 d. At each time point birds using the perches were counted. Separately, all birds were classified into a behavioural category: eating, active, drinking or resting, the latter of which included perching birds. At 35 d, nine visually male birds were selected/pen for latency to lie (LTL) testing, after which they were scored for hock burn (HB), footpad dermatitis (FPD) and leg symmetry (LS). At 42 d, nine different visually male birds were selected/pen for the same scoring before being euthanased, scored for tibial dyschondroplasia (TD), detached femoral caps (DFC) and had one toe collected for bone ash (BA). Analysis was performed using IBM SPSS Statistics version 24.

For all perch treatments, perching peaked in week 3 and declined thereafter. Perching rate (average % of population perching at any given time point) was influenced by availability of perch space, with treatment C inducing the highest rate ($P = 0.005$). Perches had no effect on other activity categories. There was no effect of treatment on weight gain, FCR or litter moisture, but notably litter pH was lowest in treatment C ($P = 0.002$). There was no effect of treatment on physiological observations (HB, LS, TD, DFC, BA) except for FPD, where prevalence was lowest in treatment C at both 35 d ($P = 0.011$) and 42 d ($P=0.037$). No significant differences were observed in LTL at 35 d, but at 42 d control birds (treatment D) had the longest LTL ($P = 0.059$), followed by treatments A, B and C respectively.

Despite more perch space reducing FPD, no benefit of perches was seen in bird LTL, suggesting that perches may not enhance bird mobility, as reported by Phibbs et al. (2019) but contrary to Groves and Muir (2013). Further research into perch space and the impact of perches on leg strength in current day meat chickens is required to clarify these results.

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THE EFFECT OF ELECTROLYTE SUPPLEMENTATION ON BEHAVIOUR AND PERFORMANCE OF BROILERS EXPOSED TO ADVERSE HIGH TEMPERATURE FOR ONE DAY PRIOR TO TRANSPORT AND PROCESSING

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Summary

The present experiment was designed to investigate the effects of electrolyte supplementation in alleviating the harmful effects of high temperature for one day prior to transport and processing. Day-old Cobb-500 broiler chicks were randomly distributed across 36 pens in a tunnel-ventilated shed for 6 weeks. From 41-42 d of age, three treatments were applied; 1: control: tap water only, 2: ES-A (in house electrolytes): 157g NaCl, 171g $NaHCO_3$ and 88.4 KCl dissolved in 100 L of tap water; 3: ES-B commercial electrolytes supplied at the recommended rate of 200g/100 L of tap water. The average temperature was 29.8°C and humidity 63.9%. Behaviour, body weight, feed intake, water intake and processing losses and carcass yields were measured. The sudden heat exposure for one day resulted in 12.7% mortalities or removals. During the heat stress, birds showed less feeding, drinking, preening, wing and leg stretching and floor pecking with more panting, crouching, walking and standing that are indicative of poor welfare, restlessness, and stress. The electrolyte supplementation for one day did not improve broiler performance and/or carcass weight on the last day of production.

I. INTRODUCTION

Heat stress is considered one of the important factors affecting the profitability of the poultry industry in hot climates. Significant economic losses occur due to high mortality, impaired feed efficiency and poorer growth rates (Lara and Rostagno, 2013). Periods of high temperature prior to transport and processing can further compound these adverse effects. One of the physiological consequences of heat stress in broilers is disruption of acid base balance. Electrolyte supplementation is a possible strategy to improve broiler welfare and performance during heat stress. The present experiment investigated the effects of electrolyte supplementation in alleviating the harmful effect of high ambient temperature for one day prior to transport and processing.

II. METHOD

Day-old mixed sex Cobb-500 broiler chicks were obtained from a commercial hatchery and transported to the poultry research facility at the University of Sydney. The birds were reared in 36 floor pens, 3 blocks of 12 pens, in a tunnel-ventilated shed. The birds were fed *ad libitum* a starter diet (1-14 d), grower (15-25 d) and finisher (29-42 d) all formulated to commercial specifications. Birds were identified with leg bands. Stocking density was 36 kg/m^2 at 42 d of age. At 41 d of age, pens were randomly allocated to one of three treatments with 4 replicates of each in the 3 blocks. Treatments were; (1) control: tap water only, (2) ES-A (in house electrolytes): 157g NaCl, 171g $NaHCO_3$ and 88.4 KCl dissolved in 100 L of tap water; (3) ES-B, commercially available electrolytes at the recommended rate of 200 g/100 L of tap water.

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All treatments were applied from 1600 h on d 41 to 1600 h on d 42 of age. The shed temperature remained at 20-22°C from 1700 h to 0800 h (d 41-42) and then at 0800 h increased gradually to reach a maximum of 32°C at 1100 -1400 h and was then reduced to 20-22°C at 1600 h. Body weight gain, feed and water intakes were determined over the 24 h from 1600 h on d 41. Temperature and humidity were recorded every 15 minutes with 7 monitors distributed along the length of the tunnel-ventilated shed. The thermal-humidity index (THI) integrates temperature and humidity into a single measure which can then be used to predict the potential severity of the environmental conditions. THI was calculated using the following equation (sourced from Progressive Dairy 2018); THI = $Tdb - [0.55 - (0.55 \times RH/100)] \times (Tdb - 58)$ where *Tdb* (dry bulb temperature) is included as °F. Severity of heat stress is determined as mild when THI is 72-79, moderate when 80- 89 and severe when > 90.

Bird behaviour was recorded using digital cameras and analysed by the scanning method of observation for three 2 h sessions on d 42 (before, while the temperature was increasing and at maximum temperature), All observations were expressed as the percentage of birds in a pen that were performing the same behaviour at a certain time. The observed behaviour patterns were feeding, drinking, crouching, standing, walking, preening, wing and leg stretching, floor pecking and panting (Reiter and Bessei, 2000). At 1930 h, birds were transported to a commercial processing plant and held in lairage overnight. At the processing plant, birds were weighed at 0600 h and processed at 0900 h. Carcass weights were taken after evisceration and before entering the chiller. These data were used to determine weight loss during transport, processing losses and carcass yields. The behavioural data were analysed using two-way ANOVA function of Gen Stat edition 18th, with the main effects being treatment and time. The growth performance and processing losses were analysed by REML linear mixed model function of Gen Stat edition 18th and the fixed effects were treatment and sex and random effects were block and pen. Significance level was ($P < 0.05$).

III. RESULTS AND DISCUSSION

On the d of high temperature, the average temperature was 29.8 °C and the average humidity was 63.3%. The range of THI was 78.4 to 93.6 and during the 3 hottest h, the THI averaged 92.9 which indicates that the birds were subjected to severe heat stress. During the high temperature treatment, 12.7% of the birds were removed as mortalities or for being at risk of severe stress according to the animal ethics guidelines.

Poultry behaviour can be indicative of welfare status. The percentage of time that individual behaviour was performed is shown in Table 1. Birds supplied with ES-A had significantly lower feeding behaviour compared to the control and ES-B birds. The feeding behaviour dramatically decreased as the temperature increased and was minimal during the hottest period. Birds may try to reduce their feed intake during periods of heat stress to reduce the metabolic heat load (Yahav et al., 1995). The drinking behaviour was not affected by treatment but was significantly affected by temperature as the birds drank more when the temperature was increasing but less during the hottest period. These results agreed with Li et al. (2015) who found that drinking behaviour increased during heat stress but the lower consumption during the hottest period is contrary to what was expected.

Crouching behaviour was not affected by treatment, but significantly influenced by the period of hottest temperature when birds spent more time crouching ($P < 0.05$). It is proposed that more time crouching allows the birds to increase heat loss by dissipation from the breast and pelvic to the litter (Mack et al., 2013). Treatment effects on preening behaviour were not significant ($P = 0.07$) with birds supplied with as ES-A tending to preen more. Moreover, there was a large drop in preening activity during the hottest temperature period. Decreased preening has been considered as indicative of poor welfare (Lara and Rostagno, 2013). Birds try to

regulate their body temperature by increasing their evaporative heat loss through panting. Panting tended (P = 0.06) to be lower for the ES-B treated birds. Elevation of temperature significantly increased panting behaviour and it was maximal during the hottest period which is supported by Ahmad et al., (2008). Treatment had no effect on resting, exploratory behaviour and/or other movement activity. Meanwhile, the walking and standing behaviour increased as the temperatures increased and is indicative of restlessness (Lin et al., 2015).

Table 1 – The proportion of broiler chickens behaviour (%) supplemented with electrolytes ES-A or ES-B for the day before processing and when exposed to high temperature (increasing, during). Control birds received tap water only.

	Treatment				Time			
	Control	ES-A	ES-B	P value	Before	Increasing	During	P value
Behaviour patterns								
Feeding	4.13^{a}	2.81^{b}	4.66^{a}	0.010	7.35^{a}	2.99^{b}	1.25^{c}	<.001
Drinking	10.15	10.29	10.99	0.431	11.44^{b}	13.14^{a}	6.86^{c}	<.001
Crouching	77.31	79.12	76.73	0.182	75.49^{b}	74.70^{b}	82.97^{a}	<.001
Standing	3.56	2.56	3.11	0.111	2.27^{b}	3.67^{a}	3.28^{a}	0.011
Walking	4.85	5.22	4.51	0.578	3.45^{b}	5.49^{a}	5.64^{a}	0.002
Preening	4.24	3.98	5.27	0.072	7.16^{a}	4.58^{b}	1.75^{c}	<.001
Wing & leg stretch	1.21	1.29	1.25	0.967	1.67^{a}	1.52^{a}	0.57^{b}	<.001
Floor pecking	2.08	3.20	2.69	0.075	1.74^{b}	3.79^{a}	2.44^{b}	<.001
Panting	18.41	17.13	15.89	0.066	0.15^{c}	12.84^{b}	38.43^{a}	<.001

a,b Within a row values with different superscripts are significantly different (P < 0.05)

Bird performance is given in Table 2. At the start of electrolyte supplementation (d 41) and end of supplementation (d 42) all the birds had similar live weight (LW). Treatment had a significant effect on feed intake with it being lower for birds supplemented with ES-A (P < 0.05). The birds supplemented with ES-A had lower live weight gain (LWG) than the control birds (P < 0.05) while the difference between the control and ES-B birds just failed to meet significance. However, 28% of all the birds had lost weight and these had no opportunity to recover from the heat stress because they were taken off food and water during transport and lairage.

Table 2 - The performance of broiler chickens supplemented with electrolytes ES-A or ES-B for the day before processing and when exposed to high temperature. Control birds received tap water only. Values are given as mean ± SEM.

	Treatments			
	Control	ES-A	ES-B	P value
Initial LW* d 41 (g)	3041 ± 27	3055 ± 27	3063 ± 27	T = 0.777
Final LW d 42 (g)	3064 ± 28	3082 ± 27	3099 ± 27	T = 0.404
LWG ** d 41-42 (g)	53 ± 17^{a}	24 ± 12^{b}	31 ± 12^{ab}	T = 0.040
Daily Feed intake (g)	164 ± 3^{a}	152 ± 3^{b}	163 ± 3^{a}	T = 0.023
Feed to gain ratio	4.38 ± 0.71	5.67 ± 1.33	5.31 ± 1.11	T = 0.616
Daily water intake (mL)	361 ± 17	370 ± 16	348 ± 16	T = 0.666

$^{a-b}$ Within a row values with different superscripts are significantly different (P < 0.05).
*LW – Liveweight.
** LWG – Liveweight gain
T – The P value for treatment effect

The transport and processing weight losses and carcass yields are presented in Table 3. There was no effect of the supplements on the transport weight loss, eviscerated carcass weight and carcass yields.

Table 3 - The processing losses of broiler chickens supplemented with electrolytes, ES –A and ES-B for one day before processing. Control birds received tap water only. Values are given as mean ± SEM.

	Treatments			P value
	Control	ES-A	ES-B	
*Transport LW loss (%)	3.57 ± 0.22	3.29 ± 0.22	3.52 ± 0.23	0.647
Eviscerated weight (g)	2463 ± 24	2451 ± 24	2448 ± 26	0.265
**F-P Carcass yield (%)	79.3 ± 0.2	79.6 ± 0.2	78.9 ± 0.2	0.132
`#A-P Carcass yield (%)	82.0 ± 0.2	82.3 ± 0.2	81.7 ± 0.2	0.195

Transport LW loss (%) - the LW loss calculated as the percentage of change from the final farm LW to that recorded prior to processing

**F-P loss (%) - the LW loss calculated as the percentage change from the final farm LW and the carcass weight following evisceration (the processed weight).

#A-P loss (%) - the weight loss calculated as the percentage of change from weight prior to processing and carcass weight following evisceration (the processed weight)

IV. CONCLUSIONS

The electrolyte supplementation for one day of high temperature prior to transport and processing was not sufficient to improve performance in the last day of production as it had limited effects on behaviour and performance. The ES-A supplemented birds had less feeding behaviour and feed intake and subsequently lower LWG. Overall, all treatments had lower LWG with no effect on feed conversion ratio, water intake, processing loss and carcass yield.

During the high temperatures, the birds made behavioural shifts in trying to cope with the heat stress by increasing their drinking (for a limited time), panting and crouching behaviour instead of feeding, preening, floor pecking and wing and leg stretching suggesting the birds experienced poorer welfare under the heat stress conditions.

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RESPONSE OF MEAT CHICKENS TO ARGININE IN REDUCED PROTEIN DIETS

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Published literature indicates that L-arginine (L-Arg), guanidinoacetic acid (GAA) and L-citrulline (L-Cit) can all provide arginine activity in broiler diets (Su and Austic, 1999; Dilger et al., 2013; DeGroot, 2018) and all are commercially available. GAA has been reported to have 77% arginine equivalence for feed conversion (Ringel et al., 2013). This study was conducted to determine the effect of arginine deficiency on performance in reduced protein diets and the efficacy of adding it back as either L-Arg, GAA or L-Cit. Day old male Ross 308 birds (n = 768) were assigned to 8 dietary treatments using a completely randomized design: normal protein (NP, CP levels of 24.9, 23.7 and 21.4% for starter, grower and finisher, respectively), reduced protein deficient in Arg (RP, CP levels 5% lower than those of NP diets for all feeding phases) and RP with 2 levels of L-Arg (238 and 476 g/kg), GAA (309 and 618 g/kg) or L-Cit (238 and 476 g/kg). Requirements for digestible amino acids were based on Ross 308 specifications. All diets were based on wheat, sorghum, soybean meal and canola meal, with enough meat and bone meal added to satisfy the requirement for available-P. Xylanase was included to the diets at 2000 BXU/kg. Feed and water were provided *ad libitum* throughout the study. Three feeding phases were applied: starter (d0 to 10), grower (d11 to 24) and finisher (d25 to 35) with 6 replicates of 16 birds (starting) per treatment in floor pens with wood shavings as litter. Compared to NP diets, birds fed RP diets had reduced feed intake (FI) (2607g vs. 3611g, $P < 0.001$), body weight gain (BWG) (1520g vs. 2607g, $P < 0.001$) and increased FCR (1.701 vs. 1.386, $P < 0.001$) from d 0 to d 35. Additions of L-Arg or L-Cit at both levels resulted in increased BWG (maximum 558g and 702g for L-Arg and L-Cit, respectively) and reduced FCR (maximum 0.242 and 0.215 points, respectively) compared to the RP diet ($P < 0.05$). Birds fed GAA had lower FCR ($P < 0.05$) but not higher BWG ($P > 0.05$) compared to the RP diet. Birds fed the high L-arginine and high L-citrulline levels had no difference in FCR compared to NP birds ($P < 0.05$). Those fed either level of GAA had lower BWG and higher FCR compared to NP ($P < 0.05$). The recommended level of GAA is 60g/kg feed. In this study, birds fed the lower level of GAA had higher FI (2879g vs. 2517g), BWG (1844g vs. 1642g) than those fed the high GAA level, suggesting possible toxicity of GAA at the higher level. The results of the current study indicate that reduction of 50 g/kg CP is excessive in practical Australian diets based on wheat, sorghum and MBM possibly due to deficiencies in phenylalanine and/or tyrosine or non-essential amino acids. Responses to added L-arginine and L-citrulline were large and significant in arginine deficient RP diets.

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AMINO ACID LINEAR REGRESSION EQUATIONS FOR AUSTRALIAN GRAINS

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Protein is the second most expensive nutrient in animal feed formulation, after energy. Diets which have excess crude protein can overload the gastrointestinal tract with excess amino acids and undigested protein (Apajalahti and Vienola, 2016), resulting in impaired feed efficiency, health and welfare issues as well as negative environmental impacts via excess nitrogen excretion. Reducing dietary protein in poultry diets can help to mitigate some of these negative effects. In poultry diets, grain can contribute up to half of the dietary crude protein; therefore, to reduce dietary crude protein, the amino acid profile of Australian grains must be comprehensive and accurate to reduce diet costs via unnecessary inclusions of expensive raw materials such as soybean meal as well as crystalline amino acids.

Raw material samples for major Australian grains (wheat, barley, sorghum and oats) were collected from across Australia for a robust dataset. Barley (n = 285), oats (n = 163), sorghum (n = 158) and wheat (n = 381) samples were scanned using a Bruker NIRS and processed using Evonik AMINONIR© Advanced 3.0. The data were analysed using SPSS statistical package (v. 24.0.0.0) and regression equations were created. Pearson correlation coefficients and interpretations of relationship strength between the two methods were based on guidelines of a strong relationship defined as $R^2 > 0.5$ and significance was accepted at $P < 0.05$.

The resulting regression equations using Australian data were compared to previously published equations from Evonik AMINODat® 5.0 developed from a global database. Discrepancies between the two equation databases establishes the necessity of using Australian specific data. When the regression equations were compared to the current Ridley database, minimal differences were observed. Exceptions to this were observed at the extremities of the protein range sampled for all grains (both high and low protein), as grains of this nature are uncommon. The resulting database of 19 proteinogenic amino acids that has been developed will better prepare the Australian poultry industry for future reductions in dietary protein and refine the use of supplemental amino acids, enabling more accurate diet formulation to improve current production and maintain performance.

Table 1 - Correlation between amino acids and crude protein in common Australian grains.

	Met	Lys	Thr	Val	Ile	Arg	Leu	Phe	Gly	Ser	Pro
Barley	0.97*	0.96*	0.99*	0.99*	0.99*	0.98*	0.99*	0.99*	0.98*	0.99*	0.99*
Oats	0.95*	0.97*	0.98*	0.98*	0.98*	0.96*	0.97*	0.96*	0.98*	0.98*	0.82*
Sorghum	0.74*	0.56*	0.90*	0.82*	0.93*	0.81*	0.69*	0.92*	0.78*	0.96*	0.68*
Wheat	0.96*	0.91*	0.99*	0.99*	0.99*	0.96*	0.99*	0.99*	0.98*	0.99*	0.98*

*P < 0.001

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EFFECT OF PROCESSING TECHNIQUE ON THE NON-STARCH POLYSACCHARIDE CONTENT OF CANOLA MEAL

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Non-starch polysaccharides (NSP) have a considerable impact on digestibility in broilers, dictating nutrient availability and energy utilization. The high NSP content of the hulls in canola meal are one factor limiting its effective use in poultry diets. The major NSP present in canola meal include cellulose, pectic polysaccharides and a variety of non-cellulosic polysaccharides, including arabinoxylans (AX). Attempts have been made to increase utilization of canola meal through the use of NSP-degrading enzymes, including xylanase (Kocher *et al.*, 2000; Mushtaq *et al*, 2007), but there have been mixed responses. Processing method influences the nutritive value of canola meal, including its NSP composition. The aim of this study was to compare the soluble and insoluble NSP and AX content of solvent-extracted, expeller-pressed and cold-pressed canola meal. Twenty-one samples of solvent-extracted, thirteen samples of expeller-pressed and eighteen samples of cold-pressed canola meal were collected from across Australia. Soluble and insoluble NSP and AX were analysed by an enzymatic-chemical method, involving determination of the released constituent sugars by gas chromatography.

Table 1 - Soluble and insoluble non-starch polysaccharide composition of solvent-extracted, expeller-pressed and cold-pressed canola meal (g/100g).

	Solvent-extracted	Expeller-pressed	Cold-pressed	*P*-Value
Soluble NSP	1.08[b]	0.69[c]	1.49[a]	<0.001
SEM	0.03	0.05	0.03	
Soluble AX	0.32[b]	0.15[c]	0.40[a]	<0.001
SEM	0.01	0.02	0.03	
Insoluble NSP	10.11[b]	7.46[c]	13.99[a]	<0.001
SEM	0.32	0.40	0.54	
Insoluble AX	5.19[a]	3.37[b]	5.24[a]	<0.001
SEM	0.09	0.09	0.13	

Processing technique diversely impacted NSP and AX composition (Table 1), suggesting susceptibility to NSP-ase degradation varies greatly depending on the quantity and specific polymer composition of the canola meal fed. The greatest abundance of soluble and insoluble NSP and AX was observed in the cold-pressed canola meals, possibly due to lessened heat damage to the polysaccharides or dilution effect of the oil. There was also more variability within the cold-pressed canola samples, suggesting that this technique results in a less consistent fibre content compared to the other two. Therefore, the necessity for NSP-ase application to combat the anti-nutritional effects of NSP, particularly insoluble NSP on encapsulating nutrients, is comparatively greater in birds fed this form of canola meal. Solvent-extracted and cold-pressed canola had very similar soluble and insoluble AX content, suggesting their response to xylanse is alike, although bird performance is generally enhanced in birds fed cold-pressed canola. Interestingly, the expeller-pressed canola meal presented the lowest NSP and AX content, as opposed to the solvent-extracted samples as predicted, possibly due to the higher heat application and oil content in expeller meal. This study highlights the need for heightened focus on the impact of processing technique on the NSP composition of canola meal and its resulting impact on NSP-ase efficacy.

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IN VIVO-BASED NIRS PREDICTIONS ROUTINELY APPLIED TO SOYBEAN MEALS: WHAT DO WE LEARN FROM A HUGE DATA SET?

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Soybean meal (SBM) remains the most frequently used protein source in monogastric feeds. Included at a rather high rate, particularly in broiler feeds, it weighs heavily on feed cost and on animal performances. It is therefore crucial for feed formulators to properly assess SBM's nutritional quality. Variability affects nutritional SBM value, depending on the geographical origin of the beans (Garcia-Rebollar et al., 2016) but also largely on the process and conditions used to extract soybean oil (Karr-Lilienthal et al., 2005). This impacts amino-acids (AA) digestibility but also energy content of SBM. This digestibility aspect cannot be easily and/or accurately checked through usual quality control plans, including indirect methods. Based on Near Infrared Spectroscopy (NIRS), the Precise Nutrition Evaluation on-line platform (PNE by ADISSEO) service enables nutritionists to have access to predictions that have been calibrated from *in vivo* measurements.

A total of 20.192 SBM spectra have been submitted under the PNE platform during the year 2018, and their predicted analytical results gathered for this study. Previously ground, all SBM were scanned on standardized and validated NIRS machines – located in 54 countries around the world - for practical quality control usage at feed mill level. In most cases, SBM production origin was not mentioned, preventing any traceability. All SBM have been predicted for their concentrations in proximate parameters (PROX) : dry matter (DM), crude protein (CP), Ash (ASH), Crude Fiber (CF) and Fat (FAT), in total AA (TAA) and for poultry digestibility coefficients of amino acids (DAA) and apparent metabolizable energy corrected for zero nitrogen balance (AMEN) using prediction models developed and validated beforehand using NIRS. In average, SBM extracted from the PNE data base contains (in g/kg DM): 524 CP / 22 FAT/ 48 CF/ 70.9 % ASH/ 32.96 Total Lysine. Beyond average values, heterogeneity is present. Coefficients of variation (CV %) reached 30% for FAT or 20% for CF content and 2.7 % for CP. Regarding the TAA content, it varies between a 3 to 4% range, the highest being for total cystine (6.2%), the lowest (2.6%) for total lysine. In the case of predicted DAA, the corresponding CV values range from 1.5% for DARG to 4.3% for DCYS. The average DLYS (86.7% ± 2.3%) appears below tables values and poorly correlated (r^2 = 0.12) with the total Lysine content. AMEN presented a 4.9% CV for an average value of 9.86 MJ/kg DM (range from 7.5 to 11.5 MJ/kg DM) ± 0.48 MJ/kg DM.

The contribution of predicted parameters to the global variability was assessed by Principal Components Analysis (PCA) (Lê et al., 2008). 3 variables together explain 86% of the total variability of SBM populations: DAA, CF and AMEN, the highest part (75%) being supported by DAA, when both AMEN and CF factors - negatively correlated – contribute to the remaining 11%. Taking the benefit of this huge data set, it has been demonstrated that digestibility parameters can be routinely applied to quality control at feed mill level to monitor SBM variability. DAA appeared to be the most efficient factor to discriminate nutritional SBM profiles, that will impact their individual economical value.

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ENHANCING NUTRIENT UTILISATION, GROWTH PERFORMANCE, GUT FUNCTIONALITY AND MEAT QUALITY OF BROILER CHICKENS THROUGH MULTI-ENZYME SUPER-DOSING

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Poultry meat is a particularly important protein source as broilers have a high feed efficiency and short production cycle compared to other animal products, making it an affordable, nutritious source of animal protein (Marangoni et al., 2015). Therefore, strategies to improve production performance of broilers will require significant research as this is key to future food security. One nutritional strategy to improve sustainability of poultry production systems is improving the efficiency of feed utilisation via the addition of exogenous enzymes into broiler diets for a cost-effective production system. Identifying the effective enzyme inclusion-rate is vital to ensure that farmers get the best performance and return on investment. This study aimed to identify the optimal dose-rate of a multienzyme (Natuzyme) at three energy levels, based on production performance, gut morphology and meat quality. A 3 x 4 factorial design of 12 dietary treatments (576 birds, 6 replicates/treatment, and 8 birds/pen) was implemented over a 42-day broiler growth trial, with weekly weights and performance data recorded. Diets were wheat-corn-soybean meal at three levels (no reduction, -0.63 and -0.84 MJ/kg) and four enzyme inclusion levels (0, 350, 700, and 1000 g/ton). One bird per pen was slaughtered at 42 days (n=72) for sample collection. Meat quality analysis was performed using breast meat, where pH, meat colour, water holding capacity, and cooking loss were measured 18 hours post-mortem. Gut morphology was studied for villi abundance, crypt depth, height, and presence of blood.

Performance data revealed that addition of Natuzyme mitigated the negative effect of energy reduction ($p \leq 0.0001$) on feed conversion ratio. Reducing dietary energy content increased average daily feed intake (ADFI) ($p \leq 0.0001$) to compensate for the energy deficiency, thus increasing the feed conversion ratio (FCR), while adding enzyme to the standard diet improved the feed conversion ratio ($p \leq 0.05$) and average daily gain (ADG) ($p \leq 0.05$). The meat quality analysis revealed that there was no difference in meat quality among treatment groups, indicating that there was no effect of multienzymes and dietary energy reduction on meat quality. The gut morphology results found that there was no significant difference among treatment groups regarding villi height, crypt depth, and goblet cell abundance. However, super-dosing (1000g/t) in the standard diet created bloody lesions in the small intestine, indicating potential damage to the intestinal wall caused by the high dose of protease which may have begun digesting the intestinal wall. In conclusion, super-dosing with multi-enzymes in reduced energy diets can improve performance parameters and thus profitability for producers and improve sustainability of production at a dose of 350g/t. However, the concentration of protease in the multienzyme may need to be altered to protect the gut lining from potential damage.

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EFFICACY OF DIFFERENT ZINC SOURCES IN BROILER PRODUCTION

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Zinc (Zn), as an essential trace element, is a common feed additive for food producing animals. Zn is supplemented in either an inorganic or organic form. Literature suggests inconsistency among both sources regarding trace mineral availability and influence on animal performance, but organic trace minerals are considered to be advantageous (Schlegel et al., 2013). Between organic trace minerals, the chemical bond is the main difference. While mono-glycinates are complexes with one amino acid (AA) bound to a metal ion, bis-glycinates are chelates with two AAs bound to a metal ion. Two studies were conducted to determine the efficacy of different Zn sources in broilers. In Study I, 640 day-old male broiler chickens (Ross 308) were randomly allocated to one of five treatments (8 replicates; 16 animals/replicate; kept in cages): I-NC: no added Zn, I-$ZnSO_4$: 80 mg/kg Zn from $ZnSO_4$, I-MG: 40 mg/kg Zn from Zn mono-glycinate, I-BG: 40 mg/kg Zn from Zn bis-glycinate, I-Combo: 40 mg/kg Zn from $ZnSO_4$ + 20 mg/kg Zn from Zn bis-glycinate. Diets were based on corn, barley and soybean meal. At day 35, body weight and weight gain were numerically, but not statistically, lower in birds of the I-NC group than in Zn supplemented birds ($P > 0.05$). The highest body weight was observed in I-Combo, followed by I-BG, I-MG, I-$ZnSO_4$ and I-NC (2322, 2297, 2294, 2280, 2262 g, respectively). FCR did not differ ($P > 0.05$) between the treatments I-NC, I-$ZnSO_4$, I-MG, I-BG and I-Combo (1.49, 1.47, 1.45, 1.44, 1.45, respectively). In Study II, 210 day-old male broiler chickens (Ross 308) were randomly allocated to one of five treatments (21 replicates; 2 animals/replicate; kept in cages): II-NC: no added Zn, II-ZnO: 100 mg/kg Zn from ZnO, II-MG: 50 mg/kg Zn from Zn mono-glycinate, II-BG: 50 mg/kg Zn from Zn bis-glycinate, II-Combo: 50 mg/kg Zn from ZnO + 25 mg/kg Zn from Zn bis-glycinate. Diets were based on wheat, soybean meal and corn. At day 35, body weight and body weight gain were numerically, but not statistically, lower in birds receiving II-NC than in birds fed Zn supplementations ($P > 0.05$). The highest body weight was observed in II-BG, followed by II-Combo, II-MG, II-ZnO and II-NC (2262, 2237, 2230, 2159, 2141 g, respectively). FCR did not differ ($P > 0.05$) between the treatments II-NC, II-ZnO, II-MG, II-BG and II-Combo (1.34, 1.32, 1.32, 1.33, 1.34, respectively). Both trials showed a numerically better performance of birds fed with an organic trace mineral source. Compared to inorganic trace minerals, bis-glycinates may assist in supporting performance in poultry, while allowing a lower inclusion rate and may therefore contribute to a sustainable poultry production. A similar effect has been shown in piglets, where half the dosage of Cu from bis-glycinate led to the same performance as in animals fed the full dosage from $CuSO_4$ (Davin et al., 2019). Therefore, the environmental impact is less.

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EFFECT OF DIETARY PEA SUPPLEMENTATION ON HEAT INCREMENT AND NET ENERGY IN BROILERS

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Field pea (*Pisum sativum*) is a good source of protein but can also provide energy due to high starch content (Petterson et al., 1997). The slowly digestible starch in pea may improve energy utilization in broilers because of the prolonged elevated plasma glucose levels (Enting et al., 2005). Thus, partial replacement of soybean meal and wheat with pea may affect the net energy value of the diet. This experiment was performed to investigate the effect of pea in diet formulation on heat increment and net energy in broilers using open-circuit respiratory calorimetry.

Ross 308 male broilers (n=24) were used for this study. A wheat-soybean meal based diet was used as a control and the treatment diet contained 50 % pea (by partially replacing wheat and soybean meal). The diets were formulated based on the Ross 308 nutrient specifications and were iso-energetic and iso-nitrogenous with the same levels of added oil. Energy levels were adjusted and made similar in both diets by the addition of Celite (an indigestible inert filler) in wheat-soy diet so that only the effect of added dietary pea could be investigated. Birds were fed a common starter crumble from d 0-10 and then a standard grower diet thereafter. On d 21, the birds were transferred to the open circuit chambers (n=2/chamber) and adapted for four days before three days of measurements from d 25 (daily feed intake, weight gain and excreta collection) to determine total tract digestibilities of nutrients, heat increment and net energy. Each treatment was replicated six times using two identical runs with three replications/treatment in each run. T-test was used to compare the means between the two treatments. Significance level was detected at $P < 0.05$ using SPSS software v. 25.

The measured apparent metabolisable energy (AME), AMEn (AME corrected to zero N retention), AMEs (AME corrected to 50 % N retention), and net energy (NE) were higher in pea-based diet compared to wheat-soy diet ($P < 0.05$) but there were no differences ($P > 0.05$) in AME and NE intakes between the two groups. Heat production, respiratory quotient, heat increment of feed, and efficiency of utilization of AME to NE (NE:AME, NE:AMEn, and NE:AMEs) did not differ ($P > 0.05$) between the two treatments. There was no effect ($P > 0.05$) of dietary pea inclusion on the total tract digestibilities of dry matter, crude protein and ash. The total tract digestibility of starch was higher ($P < 0.05$) in the pea-based diet (97.6 %) compared to the wheat-soy diet (95.8 %) which suggests that a greater proportion of starch from pea entered the caeca and was fermented. It may also be possible that pea starch was more slowly digested leading to a longer period of exposure of the starch to amylolytic enzymes. From the present study, it can be concluded that a slowly digestible starch in pea compared to wheat starch increases dietary AME and NE but does not affect heat increment of feed and the efficiency of utilization of AME to NE in broilers. However, it should be noted that varietal differences and processing conditions of pea may affect the results and thus these potential effects should be further explored.

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DO EXCESSIVE VITAMIN D CONCENTRATIONS IMPROVE OR IMPAIR BROILER GROWTH PERFORMANCE AND BONE QUALITY?

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There has been much research recently to refine broiler requirements for calcium (Ca) and phosphorus (P), especially since the routine inclusion of phytase in broiler diets (Li et al., 2016). Phosphorus and Ca play significant roles in bone metabolism and vitamin D is also pivotal to maintaining bone integrity through the modulation of Ca metabolism (Li et al., 2017). The broiler requirement for vitamin D according to NRC 1994 is 200 IU/kg while, commercially, dietary concentrations as high as 5000 IU/kg are applied (Sakkas et al., 2018). Thus, given the genetic improvement of the current broiler and the refinement of Ca and P requirements, reassessment of the use of excessive dietary vitamin D concentration is timely.

The experiment was conducted to determine the response in growth performance, including live body weight (LBW), average daily weight gain (ADWG), average daily feed intake (ADFI), feed conversion ratio (FCR) and bone ash content including tibia ash and toe ash, of three concentrations of vitamin D3 (1600, 3200 and 6400 IU/kg diet) at different levels of ileal digestible phosphorus (IDP) and Ca. In the factorial experiment, six hundred day-old, male Ross broilers fed diets based on wheat, sorghum and soybean meal, were raised for 21 days in floor pens. Diets contained two ileal digestible P (IDP) concentrations (2.0 and 2.5 g/kg) and two Ca concentrations (3.5 and 4.5 g/kg) and each diet was supplemented with phytase. Starter and grower diets were fed from Day 1 to Day 14 and Day 15 to Day 21 respectively. Each treatment had 5 replicates and each replicate had 10 birds.

Growth performance and FCR were not affected by the IDP concentration but increasing the IDP concentration significantly ($P < 0.05$) increased toe ash content. Calcium, however, had a significant ($P < 0.01$) positive impact on LBW, ADWG, FCR and ash content but not on FCR ($P > 0.05$). In contrast, vitamin D_3 supplementation showed no effect on growth performance or ash content. However, there was a positive interaction of vitamin D_3 with IDP content ($P< 0.05$) on LBW and with Ca ($P< 0.05$) in regard to bone ash content which is generally used as a means of quantifying bone mineralization.

It appears, from this study, that the high inclusion rates of vitamin D_3 in broiler diets, used by industry, do not impair bird performance or bone health. There is an indication, depending on P and Ca status, that higher concentrations of the vitamin may be beneficial for growth and bone quality. These results again emphasise the complex interrelationships among these three nutrients and the need for more research to unravel the nuances of these interactions.

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EFFECT ON GROWTH PERFORMANCE AND NUTRIENT DIGESTIBILITY OF DIFFERENT MICROBIAL LIPASE SUPPLEMENTATIONS WITH ADDED EMULSIFIER IN A LOW ENERGY BROILER DIET

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Dietary fat and oil are potential alternative energy sources for fast growing broiler chickens (Meng et al., 2004). Nevertheless, hampered fat digestion and absorption were reported in young broiler chickens with incompletely developed digestive tract (Al-Marzooqi and Leeson, 2000). Addition of emulsifier and lipases to a diet is one strategy to improve energy utilization and subsequent growth performance in broiler chickens when fed a high fat diet (Siyal et al., 2017). Studies demonstrating growth performance benefits with the addition of microbial lipases and emulsifiers in broiler chickens are limited. Therefore, the objective of the present study was to examine the effects of supplementation of two different microbial lipases and emulsifier in combination, into a reduced energy diet, on growth performance and nutrient digestibility in broiler chickens from hatch to 35 days.

Two hundred and seventy one-day-old Ross male broiler chickens were randomly allocated to 30 pens. Each pen was assigned to one of five treatments to give six replications with nine birds in each cage. Five dietary treatments were; 1) Standard (STD) diet formulated to meet the Ross 308 nutrition specification without adding any emulsifier or lipases, 2) negative control with 100 kcal/kg lower energy than STD (NC), 3) Emulsifier treatment (EMF): NC + 0.1% Polysorbate-20 and 4) Lipase treatment 1 (TLL): EMF + 0.1% TLL (*Thermomyces lanuginosus lipase*), 5) Lipase treatment 2 (CRL): EMF + 0.1% CRL (*Candida rugosa* Lipase). Corn and soybean-meal-based control diets containing beef tallow were formulated to meet the Ross 308 nutrition specification. Diets were provided on an *ad-libitum* basis in a mash form. Emulsifier and lipases were top-dressed onto the basal diet. Growth performance was measured on days 21 and 35. Ileal digesta was collected on day 35 to measure the nutrient digestibility. Our results revealed that NC lowered ($P < 0.05$) the body weight and average daily gain of the broilers compared to broilers fed STD diet from hatch to 35 days. Broilers fed CRL showed higher body weight and average daily gain compared to NC and TLL fed broilers from hatch to 35 days. Moreover, broilers fed CRL tend to have improved ($P<0.1$) feed efficiency compared to the broilers fed other treatment diets from hatch to day 35. Improved crude fat and energy digestibility ($P < 0.05$) were observed in broilers fed with CRL compared to NC fed broilers on day 35. In conclusion, our results indicated that CRL have the ability to maintain growth performance and nutrient digestibility of broiler chickens by curtailing the negative impact of low energy in the diets.

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PHOSPHORUS DIGESTIBILITY BY A RECENTLY ISOLATED PHYTASE

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Phytase supplementation of broiler diets can improve energy, amino acid, calcium (Ca) and phosphorus (P) utilization. Both fungal- and bacterial-derived phytases are commonly added to broiler diets. Phytases from different sources and even from the same source can have different pH optima, heat stability, and catalytic properties (Dersjant-Li et al, 2015). Furthermore, when different microbial phytases are included in diets, their efficacy *in vivo* can vary in comparison to their assayed activity. Therefore, biological efficacy can only be determined by feeding studies. New phytase products are constantly being developed and the efficacy of a phytase, recently isolated from *E. coli*, was examined.

Male Ross 308 broilers were randomly allocated into 20 cages (7 birds /cage) at 23 days post-hatch and offered a sorghum (920 g/kg) bioassay diet with celite (20 g/kg) as acid-insoluble ash (AIA) that was used as indigestible marker. The four dietary phytase treatments were: Diet1 (0 phytase units (FTU)/kg), Diet 2 (500 FTU/kg), Diet 3 (1000 FTU/kg) and Diet 4 (2500 FTU/kg). Each dietary treatment was fed as mash to 5 cages. After five days, lower ileal samples were collected and pooled for each pen. AIA and P in samples were determined using the method described by Huang (2004) and Plumstead et al (2008). Apparent ileal P digestibility were calculated by using equation described by Plumstead et al. (2008).

The birds fed diets containing phytase has higher apparent ileum P digestibility ($P < 0.01$) than the control group (46%). Apparent ileum P digestibility of Diet 2 and 3 increased to a similar amount (~ 61%). However, phytase superdosing in diet 4 increased ($P < .0.01$) apparent ileum P digestibility (77%) substantially.

The standard application of phytase is normally around 500 FTU/kg. It is not clear from the literature what constitutes superdosing but applications at least 3x the standard are referred to as superdosing. In this study, 5x the standard was used and digestibility of P was significantly increased. This study demonstrates that this phytase survives the acidic conditions of the gut and other studies indicate it also survives pelleting. These characteristics and its performance *in vivo* make it a possible commercial candidate.

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PARTITIONING AND EXCRETION OF DIETARY PHOSPHORUS BY BROILERS

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Understanding the dynamics of calcium (Ca) and phosphorus (P) metabolism is an important consideration when evaluating the requirements for both nutrients. Much more is known about the control of Ca than P metabolism (Li *et al*., 2017). Calcium elimination from the body is primarily through faeces, both unabsorbed dietary Ca and endogenous Ca. Kidney Ca elimination is controlled by endocrine factors and to a lesser extent by dietary Ca. In practical terms, dietary Ca may be available but not absorbed because of the animal's Ca status, whereas a large portion of dietary P that is available will be absorbed and eliminated though the urine. Therefore, body content of P appears to be primarily regulated by urinary excretion, as there is limited control of the gut absorption of P compared to Ca. The partitioning and excretion of P was evaluated using ileal P and excreta P as surrogates for P absorption and P excretion, respectively.

Day-old, male Ross broilers were fed a starter (day 1-14) and grower (day 15-21) diets based on sorghum and soybean. The experimental diets contained the same level of Ca (10 and 9.0 g/kg diet for starter and grower, respectively) and graded levels of available P from 2.5 to 5.5 g/kg diet for starter; from 2.0 to 5.0 g/kg diet for grower in increment of 1.0 g/kg which covered a range to allow a "deficiency status" at the lowest level right through to the value recommended by NRC (1994) and current values used by industry. The study was a factorial design with or without phytase inclusion. Each treatment had 5 replicates and each replicate had 10 birds.

Phosphorus contents in both ileal digesta and excreta increased as dietary non-phytate phosphorus (NPP) increased. This resulted in an extra 19.1 and 46.9% of P being excreted from birds fed diets containing NPP of 4.5/4.0 (g/kg, starter/grower) and 5.5/5.0 (g/kg, starter/grower), respectively, compared to birds fed diets containing NPP of 3.5/3.0 (g/kg, starter/grower) without phytase supplementation. Phytase significantly reduced ileal P content (10.6 vs 12.7, $P < 0.001$), but showed no effect on excreta P content (14.2 vs 14.6, $P > 0.05$). Broilers fed on diets with lower NPP levels excreted much less P in excreta. There were more consistent and stronger relationships between dietary NPP and excreta P concentrations ($R^2 = 0.9099$) than dietary NPP and ileal P concentrations ($R^2 = 0.4912$), indicating high absorption of available P but rapid urinary excretion of absorbed P to maintain P homeostasis. A similar interpretation can be made from other published data (Leske and Coon, 2002), implying that measurement of P excretion may become the important strategy in formulating diets to meet exact P requirement and assess enzyme effectiveness in poultry.

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MATERNAL FEED RESTRICTION UPREGULATES INSULIN-LIKE GROWTH FACTOR I EXPRESSION IN 42 DAY OLD MALE PROGENY

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Environmental factors encountered during pre-natal life influence the developmental trajectory of a developing embryo, altering post-natal phenotypes (Du et al., 2015). Maternal nutrition and stress directly influence the maternal environment, with both factors prominent in the broiler breeder industry. Broiler breeders are feed restricted to improve reproductive output; however evidence suggests hens suffer from chronic stress due to prolonged hunger (Mench, 2002). Feed restriction in breeder hens is reported to reduced progeny growth rates (Hynd et al., 2016); however the mechanisms by which it does so remains unclear. Glucocorticoids have been shown to interact with various endocrine pathways, including the growth hormone/insulin-like growth factor I axis, a key regulator of growth and metabolism in poultry. Therefore a study was designed to investigate whether feed restriction in breeder birds influenced male progeny insulin-like growth factor I (IGF-I) hepatic mRNA expression and plasma concentrations. 36 broiler breeder hens where separated into three groups based on maternal body weight (bwt) (high, medium, low) at 24 weeks of age and fed accordingly to maintain their bwt until 43 weeks of age. Eggs were collected at 42 weeks of age and 170 viable chicks hatched. Progeny were housed in groups of 10, and reared together until 42 days of age, when 69 birds (23 per treatment) were euthanised. IGF-I and other related gene mRNA expression was determined by Q-PCR, whilst IGF-1 plasma concentrations were analysed via ELISA.

IGF-I mRNA expression was increased in progeny produced from low maternal bwt hens (P = 0.020), accompanied with an up regulation of IGF-I receptor (IGF-IR) expression (P = 0.097) (Figure 1), whilst no difference was observed in IGF-I plasma concentrations. However these findings suggest that maternal feed restriction and/or low maternal bwt may reduce the synthesising capacity of IGF-I during early post-hatch development.

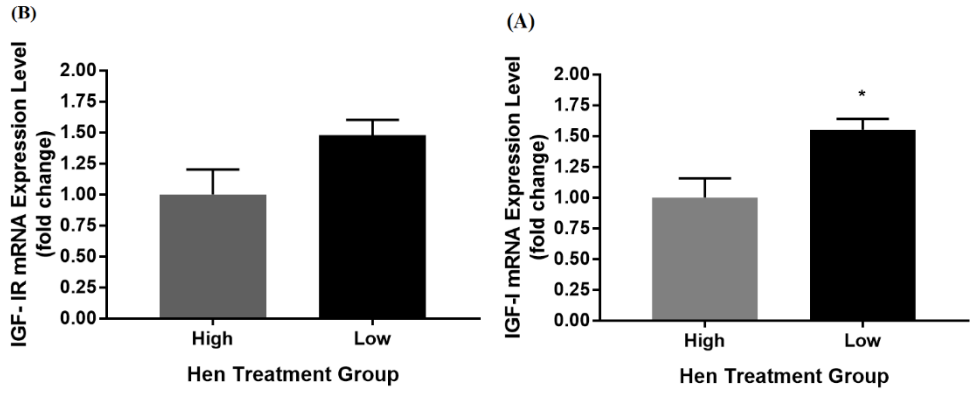

Figure 1 - IGF-I **(A)** and IGF-IR **(B)**, mRNA expression for day 42 male broiler chicken progeny produced from low maternal bwt (low) or high maternal bwt (high) breeder hens. qPCR data shown as mean fold change ± SEM. * denotes statistical significance between at the 0.05 confidence level.

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DEGRADATION OF HEXAKISPHOSPHATE IN THE GIZZARD AND ILEUM OF BROILERS FED DIETS WITH TWO CALCIUM TO PHOSPHORUS RATIOS AND PHYTASE DURING SUBCLINICAL NECROTIC ENTERITIS

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Phytate is a polyanionic molecule with the capacity to chelate Ca, forming mineral-phytate complexes. The presence of these complexes decreases the efficacy of phytase. Ca-phytate formation is dictated by molar ratios of the constituents, gastrointestinal pH and the concentration of dietary Ca relative to P (Sommerfeld et al., 2018). The hypothesis of this study was that a high dietary Ca to P ratio will reduce the ability of phytase to hydrolyze phytate, which will exacerbate necrotic enteritis (NE) in broilers. A total of 768 Ross 308 male broiler chicks were randomly distributed to 8 treatments in a factorial arrangement. Factors were: NE challenge (no or yes), phytase level (500 or 1500 FTU/kg; both using 500 matrix values for Ca (0.16 %), P (0.15 %) and Na (0.035 %; Quantum Blue^{TM}, AB Vista, Malborough, UK) and Ca level (0.6 or 1.0% Starter, 0.5 or 0.9% Grower, 0.4 or 0.8% Finisher) whilst maintaining the same level of avP (0.40 Starter, 0.35 Grower and 0.35 Finisher). There were 48 pens, 16 birds per pen and 6 replications per treatment. Half of the birds were challenged with 5000 oocysts of field strains of *E. acervulina* and *E. brunetti*, and 2500 oocytes of *E. maxima* (Eimeria Pty Ltd) on d 9, and 10^8 CFU per mL of *Clostridium perfringens* Strain EHE-NE18 (known to express NetB toxin, CSIRO) on d 14 and d 15. Gizzard and ileal digesta were collected at d 29 for determination of phytate and its inositol phosphate (inositol x-phosphate, IPx: IP3, IP4, IP5 and IP6) esters, based on the methods of Walk et al. (2018).

The NE challenge as a main effect increased the concentration of inositol (3.840 vs 2.404 µmol/g DM; $P < 0.01$) in the gizzard. Phytase inclusion at 1500 FTU/kg increased IP3 (0.870 vs 0.388 µmol/g DM; $P < 0.001$) but decreased IP4 (0.607 vs 2.038 µmol/g DM; $P < 0.001$) and IP5 (0.133 vs 0.262 µmol/g DM; $P < 0.05$) in the gizzard. Also, narrower dietary Ca:P increased inositol level (3.547 vs 2.697 µmol/g DM; $P < 0.05$) in the gizzard. In the ileum, a challenge × Ca:P interaction was detected for IP5 ($P < 0.05$), with wider Ca:P increasing IP5 both in challenged and unchallenged birds. Additionally, a phytase × Ca:P interaction was observed for ileal IP3 ($P < 0.01$), IP4 ($P < 0.05$) and IP6 ($P < 0.01$). In groups fed 1500 FTU/kg, wider Ca:P increased IP3 and IP4 compared to those fed 500 FTU/kg. The IP6 was increased by wider Ca:P both in birds offered 500 and 1500 FTU/kg phytase diets. The NE challenge as a main effect decreased IP3 (0.688 vs 1.175 µmol/g DM; $P < 0.05$) and IP6 (6.47 vs 10.49 µmol/g DM; $P < 0.05$), but it increased inositol (30.33 vs 18.70 µmol/g DM; $P < 0.001$) in the ileum. The results reconfirm that the majority of IP degradation occurs in the gizzard. Furthermore, these findings support the consensus that superdosing phytase diminishes the anti-nutritive effect of phytate and yields more soluble lower esters and inositol. In conclusion, though superdosing of phytase might increase IP destruction with narrower Ca:P, an accumulation of inositol in the ileum during NE might exacerbate the incidence.

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EFFECTS OF DIETARY SUPPLEMENTATION OF A BUFFERED FORMIC ACID AND 1-MONOGLYCERIDES ON GROWTH PERFORMANCE OF BROILERS CHALLENGED WITH SUBCLINICAL NECROTIC ENTERITIS

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Necrotic enteritis (NE) is a poultry disease of global concern mainly due to reduced growth, increased mortality, and increased veterinary and managemental costs associated with it (Wade and Keyburn, 2015). *Clostridium perfringens*, a spore-forming gram-positive bacterium, is considered to be the causative agent of NE in chickens. In the past, the common remedy to ameliorate the negative effects of NE was an addition of antibiotic growth promoters (AGP) in feed. However, with AGP phasing out worldwide, there have been efforts to find alternatives to improve intestinal health. In this regard, special attention has been paid to short and medium chains fatty acids due to their proven benefits on intestinal health and performance (Zentek et al., 2012).

A study was conducted using 816 as-hatched 1-d-old chicks (Cobb 500) to evaluate the effects of a buffered formic acid (Amasil NA™) and a synergic combination of 1-monoglycerides of short and medium chain fatty acids (Balangut™ LS P). These additives were added to wheat-soy basal diet singly or in combination at various rates and assigned to six dietary treatments with eight replicates of 17 birds per pen unchallenged negative control (NC) with no additive (T1); and five challenged groups orally inoculated with 1 mL *Eimeria* vaccine on d 9 and *C. perfringens* on d 14 with: no additives as positive controls (PC, T2); buffered formic acid (0.3% throughout all the phases, T3); 1-monoglycerides (0.5%, 0.3%, and 0.2% in starter, grower and finisher phases respectively, T4); a combination of buffered formic acid and 1-monoglycerides (0.3% + 0.3%, 0.2% + 0.2%, and 0.2% + 0.15% in starter, grower and finisher phases respectively, T5); and Zn bacitracin (0.05 % throughout all the phases, T6). Feed refusal and body weight of birds were measured in each pen every week to calculate performance parameters until d 35. On d 16, four birds (two males and two females previously feather sexed and marked) were randomly selected from each pen and euthanised for scoring NE lesions in the small intestine.

The results confirmed the presence of sub-clinical NE as observed by lower feed intake ($P < 0.01$) and weight gain ($P < 0.01$) in the challenged PC compared to the unchallenged NC. While the birds in NC had no lesions in the gut (lesion score 0), those in PC had lesions in the jejunum and ileum sections of the intestine which were significantly different from the NC ($P < 0.01$). During 9-21 d, challenge increased FCR by 8 points ($P < 0.01$) but the group supplemented with a combination of buffered formic acid and 1-monoglycerides had similar FCR to the NC ($P > 0.05$) and 5 points less FCR than the PC. During 21-35 d, there were no differences ($P > 0.05$) in feed intake and weight gain between NC and PC but the challenged groups with additives reduced FCR by 6-7 points ($P < 0.01$) compared to NC. During 0-35 d, the additives had no effect on feed intake and weight gain in the challenged groups, but FCR tended to be lowered ($P=0.08$) and the lowest FCR was observed in the group fed the combination of buffered formic acid and 1-monoglycerides. These results suggest that, under NE challenge, the dietary supplementation of buffered formic acid and 1-monoglycerides at suggested dose rates may improve the efficiency of feed utilization in broilers possibly due to improved intestinal health.

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EFFECTS OF SUPPLEMENTED OREGANO ON THE MATURATION OF BROILER FAECAL MICROBIOTA

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Summary

Oregano-based products are being increasingly used in livestock feed as a replacement for antibiotics. Despite the increasing use of oregano on poultry farms, little is known about the influence it may have on the development of the intestinal microbiota of chickens, especially when used from first days of life. We investigated the effect of oregano supplementation in the development of intestinal microbiota in Ross 308 broilers grown with and without 2% oregano in the feed. Faecal samples were collected weekly from week 1 to week 6, and microbial communities were investigated via sequencing of 16S rRNA gene amplicons. Linear regression based on Pearson's correlation showed no difference in taxa positively or negatively correlated with the timeline, in either known pathogenic or beneficial genera, despite some taxa identified as differentially abundant ($P < 0.05$) between control and oregano treatments. The results we present question the validity of using faecal samples to pinpoint slight differences in microbiota.

I. INTRODUCTION

Oregano essential oil contains many compounds, of which carvacrol, thymol and their precursors are the major components, accounting for approximately 80% of the contents. Carvacrol and thymol have been shown to actively disrupt the cell membranes of bacteria leading to cell death, promoting the use of oregano as a phytobiotic (Rao et al., 2010). Phytoadditives such as oregano are becoming more popular as organic and natural alternatives to antibiotics. Many commercial poultry feed supplements are based on oregano antimicrobial products.

Although there is evidence of the presence of bacteria in ovo, the bulk of the microbiota colonisation of the chicken gastrointestinal tract starts from hatch, resulting in a highly populated gut within three days (Lu et al., 2003; Apajalahti et al., 2016). Commercial chickens have a highly variable microbiota (Stanley et al., 2012) that could be easily influenced by various feed additives and feed ingredients during first days of colonisation.

Numerous studies have investigated the effects of oregano on human and animal pathogens. Akdemir Evrendilek (2015) reported that oregano could inhibit growth of *Listeria innocua*, coagulase-negative staphylococci, *Staphylococcus aureus*, *Bacillus subtilis*, *Yersinia enterocolitica*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Proteus mirabilis*, *Escherichia coli* O157:H7, and *Klebsiella oxytoca*. Silva et al., (2013) tested essential oils of known antibacterial herbs including thyme, oregano, rosemary, verbena, basil, peppermint, pennyroyal and mint for their activity against the food spoilage bacteria. Oregano showed the highest antimicrobial activity against the food spoilage bacteria *Listeria monocytogenes, Clostridium perfringens, Bacillus cereus, S. aureus, Enterococcus faecium, Enterococcus faecalis, Staphylococcus epidermidis, Salmonella enterica, E. coli* and *Pseudomonas aeruginosa*. Others suggested that oregano's main active ingredient, carvacrol, can control biofilm formation by disrupting bacterial quorum sensing (Burt et al., 2014).

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With the established ability of oregano to control some of the major poultry pathogens, we hypothesised that a high concentration of oregano in the feed given from day 1 would remodel gut development and prevent, or reduce, the colonisation of some major poultry pathogens such as *Salmonella* and *Campylobacter* species.

II. METHOD

Oregano and feed preparation: Dried oregano leaves (Saucy Spice Company, North Boambee Valley, NSW, Australia) were blended (100 g, 1.5 min/max, 1500W, Nutri Ninja Auto iQ Duo, SharkNinja, USA) and processed in a Planetary Ball Mill Machine (speed no. 5, 2 hrs, 40 g per each run, Changsha Yonglekang Equipment, China). The oregano was then sized using an electric sieve machine (Changsha Yonglekang Equipment, China) and the particle size of the powder was determined by laser diffraction (Mastersizer 2000, Malvern, ATA scientific, Australia); the average size was 10 µm. Antimicrobial and coccidiostat free chicken starter diet crumble (Red Hen, Laucke Mills, Australia) was formulated to meet or exceed the National Research Council guidelines for broiler chickens (NRC, 1994). The 2% of oregano (0.02 kg/kg w/w), was evenly distributed through the feed using an electric mixer (125 L mixer, Ozito, China).

Birds and management: The study was approved by the Animal Ethics Committee at Central Queensland University under the approval number 0000020312 and the data on microbiota structure of different intestinal sections (ileum, jejunum and caecum) at slaughter day at 6 week old with pathogen reduction profiles in these sections, histology and SCFA profiles have been published separately (Bauer et al, 2019). Day-old broiler chicks (Ross Broiler 308, Bond Enterprises, Toowoomba) were delivered to the experimental facility and randomly distributed into two treatments, control (n = 12) and 2% w/w oregano (n=12). All birds were fed ad libitum and had unrestricted access to drinking water. The purpose of this experiment was not to evaluate bird performance, but the development of the microbial communities. Birds were individually tagged using coloured leg rings. Feed consumption and individual bird weights were measured each week, for six weeks. Freshly voided faecal samples (not swabs) were taken weekly from each birds separately.

DNA extraction and 16S rRNA sequencing: DNA was extracted from faecal samples for 16S rRNA gene sequencing. The detailed protocol has been previously described (Gangadoo et al., 2019). The primers contained barcodes, spacers, and Illumina sequencing linkers that have been previously described (Fadrosh et al., 2014). The 16S rRNA gene sequencing library preparation and amplification followed the Illumina recommended protocol (Illumina Inc., San Diego, CA, USA). Sequencing was conducted on the Illumina MiSeq platform using 2x300 bp paired-end sequencing. The sequence data generated from each sample were initially analysed using Quantitative Insights Into Microbial Ecology (QIIME v.1.9.1) as previously described (Gangadoo et al., 2019). All OTUs with less than 0.01% abundance were removed. A Hellinger transformed OTU table was used in statistical investigation. Significantly differentially abundant taxa were identified using ANOVA, and linear regression using Pearson's correlation was also investigated. Calypso was used for further data exploration and visualization (Zakrzewski et al., 2017). The sequence data is publicly available on the MG-RAST database under project mgp89580 and library accession number mgl745316.

III. RESULTS

Oregano and the timeline of microbiota development: The overall structure of the faecal microbiota did not differ significantly between control and oregano supplemented communities (Adonis on weighted UniFrac matrix) at week 1 (P = 0.234), week 2 (P = 0.689), week 3 (P =

0.648), week 4 (P = 0.405), or week 5 (P = 0.176); however, they differentiated at week 6 (P = 0.036). The number of differentially abundant taxa at the genus level was not very high at any of the weekly data, and different genera were affected every week, for example, *Lactobacillus* was reduced in the oregano supplemented group 4.9 fold during week 3 only, and not significantly affected after that. There were also a range of pathogen-rich genera equally inconsistently reduced in oregano at multiple different sampling points, for example, *Streptococcus* was reduced only in week 1, *Clostridium* only in week 3 or *Staphylococcus* only in week 4. However, the genus correlation with the age of birds, across all 6 weeks, was not different in oregano supplemented birds when compared to the un-supplemented control. All significantly correlated genera (Pearson) changed with age in the same direction in both control and oregano supplemented birds (Table 1), significantly increasing with the birds' age in both control and oregano, or reducing in both groups. Oregano supplementation was not able to change the correlation direction or significance even in those taxa that were significantly reduced in abundance in the oregano supplemented birds at particular sampling times.

Table 1 - Pearson-based correlations of genera with the birds' age, performed separately for control and oregano birds. Correlations were not reversed or removed by supplemented oregano but instead remained in the same direction. Every genus that increased over time in the control group was also significantly increased over time in the oregano group and vice versa. Significant positive correlations are shown in italic font and negative in bold.

	CONTROL		2% Oregano	
Taxa	P-value	R	P-value	R
Arthrobacter	0.000007	-0.529	1.2E-06	-0.553
Lactococcus	8.7E-06	-0.524	1E-08	-0.631
Allobaculum	0.000026	-0.501	1.9E-06	-0.544
Bacillus	0.000027	0.499	0.000003	0.535
Turicibacter	0.000031	0.496	2.1E-06	0.542
Leuconostoc	0.000035	-0.493	2.8E-08	-0.616
Microbacterium	0.000046	-0.487	2.7E-08	-0.617
Blautia	0.000066	-0.477	0.000075	-0.467
SMB53	0.00013	0.461	1.8E-09	0.655
Lactobacillus	0.00035	0.433	0.00016	0.445
Serratia	0.0004	-0.429	6.7E-07	-0.564
Facklamia	0.0004	0.429	4E-07	0.573
Paracoccus	0.0011	0.400	0.000034	0.483
Epulopiscium	0.0016	0.387	0.0013	0.385
Leucobacter	0.0021	-0.378	0.00016	-0.445
Trichococcus	0.0022	0.377	3.2E-07	0.577
Proteus	0.0024	-0.373	0.000078	-0.463
Sarcina	0.0045	0.351	1.1E-06	0.554
Corynebacterium	0.006	0.340	0.00048	0.415
Acinetobacter	0.011	0.314	0.00033	0.425
Sphingobacterium	0.031	0.270	0.000019	0.496

IV. DISCUSSION

Oregano based products are among the most frequently used antibiotic alternatives in poultry farming with positive anecdotal feedback in terms of farmer perceived pathogen control. There is an abundance of *in vitro* studies showing that oregano can inhibit a range of common

livestock and poultry pathogens, indicative that oregano would be expected to influence the development of gut microbiota. Although the microbial communities in the excreta became significantly different by week 6, we found that oregano did not prevent the gradual increase/decline of any microbial taxa or reverse its temporal development. At best, oregano in the formulation, at the level tested here, influenced the extent of such gradual changes in microbiota which in turn resulted in slightly differential microbial communities by the end of 6 weeks of continual oregano supplementation. An interesting outcome in this study was the variability of the differential microbiota from week to week. This raises several questions regarding the reliability of significantly differential taxa when there are no significant overall community differences, as well as the importance of timing of the sampling and diet influence. A critical criterion for a phytochemical to be considered as a viable replacement for antibiotics is a minimal disturbance of the intestinal community and, ideally, high inhibitory preference towards poultry pathogens. In the current study, oregano did not disturb the microbial communities over time, even at the high dose of 2% supplementation. In the separate publication (Bauer et al., 2019) we published consistent reduction of some pathogens across gut sections (ileum, jejunum and caecum). Weekly faecal data indicate that oregano supplementation inhibited some pathogens, however not reproducibly over time, and did not exhibit any major influence on the temporal microbiota development trends. The finding that using faecal samples, different genera were affected every week may be result of well-established faecal microbiota sample variability (Stanley et al., 2015) that is a result of periodic emptying of different gut sections in chicken, temporal microbiota fluctuation or simply methodology noise. Either way, our results indicate that intestinal samples, such as caecum, are more appropriate to detect subtle differences in microbiota in chicken than are faecal samples.

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CHARACTERIZATION OF ANTIBIOTIC RESISTANCE PATTERN OF *SALMONELLA* SPP. ISOLATED FROM BROILER CHICKENS, FARMWORKERS AND ENVIRONMENT IN TWO SELECTED DISTRICTS OF BANGLADESH

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Poultry farms act as an important source of transmission of antimicrobial-resistant (AMR) *Salmonella* to the environment as well as to humans. Indiscriminate and prophylactic use of antibiotics in poultry farms is one of the reasons behind it. Therefore, a cross-sectional study was conducted to determine the prevalence of *Salmonella* in broiler chickens, farm environments, as well as farmers to identify the risk factors for *Salmonella* colonization in broiler farms. This study further aimed at characterizing the AMR pattern of isolated *Salmonella* spp. and determining the prevalence of the carbapenem resistance gene (NDM-1).

Total 150 samples comprising cloacal swabs (n=50) from broiler chickens, farm sewage (n=50) and hand wash water (n=50) of farmers along with data on farm management were collected from 50 broiler farms of Mymensingh and Gazipur districts in Bangladesh. Cloacal swabs were collected from ten birds on each farm and pooled to make one sample. Farmers' hand were washed directly in 100 ml of sterile distilled water which was then taken into a sterile tube and sealed. Voluntary and informed written consent was obtained before data collection. *Salmonella* was isolated using Xylose Lysine Deoxycholates (XLD) agar after pre-enrichment in buffered peptone water. Presumptive *Salmonella* colonies were observed by gram staining and assessed by catalase, sugar fermentation, indole, methyl red, and Voges Proskauer test. Biochemically confirmed *Salmonella* isolates were then confirmed by PCR with an Internal Transcribed Spacer (ITS) region of 16S-23S rRNA gene-specific primers as described by Chiu *et al.* (2005). Antimicrobial susceptibility testing was conducted to ten antibiotics (levofloxacin, ciprofloxacin, ceftazidime, ceftriaxone, cefotaxime, amoxicillin-clavulanic acid, colistin, doxycycline, imipenem, and meropenem) by disc diffusion technique (CLSI, 2015). Carbapenem resistance gene (NDM-1) was detected by PCR. Multivariable logistic regression was done to identify the risk factors associated with the *Salmonella* colonization in broiler chickens.

Salmonella was detected with an overall prevalence of 66% of the 150 samples. Among the three types of samples collected, the highest prevalence, 82%, was observed for cloacal swabs. Hand wash water collected from farmers exhibited the lowest *Salmonella* prevalence of 44%. *Salmonella* colonization in broiler chickens was significantly associated with ≤5 years experience of farmers (Odds ratio, OR:14.17, 95% Confidence interval, CI:1.75-114.7) and 11 to 20 day old (OR:18.55, 95% CI:1.18-292.2) as well as more than 20 day old (OR:15.71, 95% CI:1.11-222.67) birds. Overall, *Salmonella* was found resistant to colistin (88.9%), doxycycline (84.8%), ciprofloxacin (78.8%), ceftazidime (64.6%) and imipenem (36.4%). Lower resistance was observed for levofloxacin (15.2%) and meropenem (18.2%). Most importantly, 89.9% of the total isolates and almost all the cloacal swab isolates (97.6%) showed multidrug resistance (MDR). In genotypic resistance analysis, one broiler chicken isolate was found positive for the NDM-1 gene when the test was repeated three times with positive control in PCR.

High percentage of MDR *Salmonella* found in our study and the presence of carbapenem resistance gene suggest that a serious threat to public health may emerge. Further studies should be conducted with sufficient sample size to evaluate the situation.

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PREVALENCE OF ANTIMICROBIAL RESISTANT *CAMPYLOBACTER* SPP. AND THEIR RESISTANCE GENES IN CHICKENS IN TWO DISTRICTS OF BANGLADESH

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Campylobacter spp. is one of the most frequent causes of foodborne gastroenteritis having zoonotic importance, and the emergence of antimicrobial-resistant (AMR) *Campylobacter spp*. is of particular concern to public health. These potential AMR *Campylobacter* spp. could be transferred to humans through animal food, particularly chickens (Reddy and Zishiri, 2017). The present study was conducted to estimate the prevalence of AMR *Campylobacter spp*. and their antibiotic resistance genes (ARGs).

A total of 840 cloacal swab samples were collected from 84 broiler and cockerel farms (10 from each farm and pooled as one sample) from two selected districts of Bangladesh, then *Campylobacter* spp. were isolated and finally confirmed by PCR. Antimicrobial susceptibility testing was performed against 14 antibiotics from 12 different classes by disk diffusion method, and nine ARGs were screened in positive isolates using multiplex PCR. Association between phenotypic and genotypic resistances was investigated by likelihood-ratio χ^2-test using SPSS.

In the results, 40.5% (34/84) isolates were *Campylobacter spp*. of which 100% isolates were resistant to at least two of the antimicrobial compounds. Moreover, multi-drug resistance, extensive drug resistance, and possible pan-drug resistance were observed in 97.1% (33/34), 50% (17/34) and 5.9% (2/34) *Campylobacter spp*. Isolates, respectively, with 73.5% (25/34) resistance to at least 10 antimicrobial agents. With regard to individual antibiotics, the highest resistance was observed to streptomycin (97.1%) and clindamycin (97.1%), followed by ampicillin (94.1%), tetracycline (94.1%), erythromycin (91.2%), ciprofloxacin (88.2%), nalidixic acid (85.3%), imipenem (82.4%), amoxicillin-clavulanic acid (73.5%), and azithromycin (61.8%), whereas the lowest resistance was found against colistin (35.3%). In genotypic resistance patterns, *bla*TEM (97.1%) was the most prevalent gene, followed by *tetA* (70.6%), *tetB* (32.4), *qnrS* (26.5%), *bla*CTX-M-1 (20.6%), *qnrB* (11.8%), *qnrA* (8.8%), and *bla*SHV (8.8%), whereas, *bla*CTX-M-2 did not exist in any of the isolates. Among phenotypically tetracycline-resistant isolates, 75% and 34.4% were detected as *tetA* and *tetB* respectively, and only three isolates harboured both *tetA* and *tetB* genes. Moreover, the prevalence of *qnrA*, *qnrB*, and *qnrS* genes was 9.7%, 12.9%, and 29%, respectively, in fluoroquinolone-resistant isolates, while no isolates carried any two of these genes simultaneously. Of the ceftazidime resistant *Campylobacter spp*., 20% isolates had *bla*SHV, and, furthermore, all the isolates resistant to ampicillin carried *bla*TEM gene. Tetracycline, ampicillin and ceftazidime resistance in *Campylobacter spp*. were significantly ($P \leq 0.05$) associated with the presence of *tetA*, *bla*TEM, and *bla*SHV genes, respectively.

Chickens of 97% (33/34) *Campylobacter*-positive farms were administered antibiotics of which 66.7% (22/33) of farms used multiple classes of antibiotics, 51.5% (17/33) were administered antibiotics for more than seven days as prophylactic therapy, and 90.9% (30/33) of farmers used antibiotics without following any prescriptions by veterinarians. Such practices of antibiotics usage may be associated with the emergence of AMR *Campylobacter*.

The findings of the study indicate a serious threat to public health in terms of the high frequency of MDR *Campylobacter* strains and their resistance genes. This data is helpful in monitoring the emergence of multi-drug resistant *Campylobacter* in chicken production.

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